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# THE AMINO-ACID MINIMUM FOR MAINTENANCE AND GROWTH, AS EXEMPLIFIED BY FURTHER EXPERIMENTS WITH LYSINE AND TRYPTOPHANE.<sup>1</sup>

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(Received for publication, March 18, 1916.)

The current view of the construction of new tissue involves the synthesis of new protein as an essential feature. Growth will be limited, therefore, by *any* factor which prevents this synthesis. The lack of any component amino-acid which cannot be manufactured directly in suitable amounts in the body represents such a limiting factor. This explains why adequate growth has never been obtained with rations in which the nitrogenous components fail to furnish sufficient proportions of certain amino-acids such as tryptophane, lysine, or cystine. The wear-and-tear features of the metabolism of maintenance without growth may also call for a certain quota of some, if not all, of these same amino-acids.

In an earlier paper we expressed the following conclusion: "Obviously the relative values of the different proteins in nutrition are based upon their content of those special amino-acids which cannot be synthesized in the animal body and which are indispensable for certain distinct, as yet not clearly defined processes which we express as maintenance or repair."<sup>2</sup> The well known "law of minimum" may be expected to apply here as in other nutrition phenomena. In *growth* some of the amino-acids *must* become an integral part of new protein in the body; so that their participation

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>2</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, **vii**, 340.

Whether maintenance is possible when lysine is completely lacking from the diet has never been demonstrated, owing to the unavoidable presence of a little protein in the "protein-free" milk, but, in any event, the need for lysine is not comparable with that for tryptophane, for with the similar small amounts of this amino-acid available in the muscles of the animal and also in the protein of the "protein-free" milk, the animals decline rapidly on the zein foods unless tryptophane is added in sufficient quantity, which for long periods we have found to be equal to 3 per cent of the zein. Even additions of lysine to the zein foods do not prevent the decline unless tryptophane is also added.<sup>10</sup> Another record of this sort is shown in Chart I for Rat 1905.<sup>11</sup>

In the experiment just cited a unique evidence of the inhibition of growth was presented throughout the whole period of 6 months during which the rat remained at constant body weight. A small patch of hair on the animal's back had been dyed red for purposes of identification before the experiment was begun. This remained unchanged until lysine was added to the diet at the end of 182 days, and growth was resumed, when the color soon disappeared. New growth had become possible in the hairs as in other parts of the body.

The record of the experiment just referred to, for Rat 1892, is given in Chart I. In the records of two rats, 2475 and 2476, presented in Chart II, it is shown that zein food can be made adequate for maintenance and even for slight growth by additions of both lysine and tryptophane with far smaller proportionate quantities of the latter than was the case where tryptophane alone was added. We are unprepared to explain the exact significance of this conserving effect of the presence of lysine. The experiments were of comparatively short duration, having been planned to show the influence of different proportions of these two amino-acids. In Chart III are recorded results of experiments to de-

<sup>10</sup> Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325. (See Rat 1900♂, Chart VI, p. 347.)

<sup>11</sup> The sudden rise in body weight indicated in the chart just prior to the death of this animal was due to a pathological collection of fluid in the abdominal cavity. It must not be interpreted as an indication of the ability of the added lysine to render the zein food adequate for maintenance.

termine the quantitative limits for the proportions of lysine and tryptophane needed to promote good growth with zein foods. (See also Rats 2154 and 2161, Chart I.) These graphic representations of the body weight show strikingly the effect of increasing or decreasing the quantities of lysine and tryptophane. Both must be present in sufficient amount before maximal effects can be obtained. Furthermore, we have already intimated that for ideal growth zein ought perhaps also to be supplemented by additions of arginine and histidine which it yields in rather small proportions.<sup>12</sup>

The quantitative rôle of lysine is shown even more strikingly in the case of gliadin foods. When gliadin is the only protein in the ration, growth is very slow, if it occurs at all.<sup>13</sup> Gliadin yields tryptophane; and its lysine content is now estimated at about 0.92 per cent.<sup>14</sup> The adequacy of the gliadin foods for the maintenance of rats (without noteworthy growth) at various ages represented by correspondingly different body weights has been demonstrated in earlier papers. In these experiments the foods contained 18 per cent of gliadin. We have since found that foods containing only 9 per cent of gliadin can maintain the body weight of rats at least for short periods. With foods containing only 2 per cent of gliadin maintenance is no longer possible.<sup>15</sup> Gliadin can supplement zein food, so as to make the latter adequate for maintenance.<sup>16</sup> Inasmuch as isolated tryptophane acts in the same way, biological evidence for the presence of a considerable proportion of tryptophane in gliadin is thus afforded.

It has been shown that the addition of lysine equivalent to 3 per cent of the gliadin fed readily converts the gliadin food into a

<sup>12</sup> Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 1. (See Chart I, p. 11.)

<sup>13</sup> Osborne and Mendel, *Carnegie Institution of Washington, Publication 156*, pt. ii, 1911; *Z. physiol. Chem.*, 1912, lxxx, 328; *J. Biol. Chem.*, 1912, xii, 473; 1912-13, xiii, 233; *Science*, 1913, xxxvii, 185; *J. Biol. Chem.*, 1914, xvii, 325.

<sup>14</sup> Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

<sup>15</sup> Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351. (See Rats 2435 and 2463, Chart VIII, p. 377.)

<sup>16</sup> Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325. (See Rat 1113 ♀, Chart I, p. 342.)

ration suitable for growth.<sup>17</sup> More recent attempts to ascertain the lower limit of this lysine requirement are presented in Chart IV (Rats 2054, 3 per cent lysine; 2268, 2271, 2 per cent lysine; 2363, 2366, 1.5 per cent lysine; 2294, 2291, 1 per cent lysine). A distinct effect upon the rate of growth is not apparent until this lowest addendum of lysine is reached. Recalling that the gliadin itself furnishes nearly 1 per cent of lysine, it is obvious that, to produce satisfactory growth, lysine equal to somewhat over 2 per cent of the protein must be present in the food. For the purposes of comparison the food intake and gains in body weight for a period of 3 weeks of rats of comparable size, some of which are referred to in Chart IV, are summarized below:

Lysine added to gliadin food.		Rat.	Initial body weight.	Food intake in 21 days.	Gain in body weight in 21 days.
As per cent of gliadin	In 100 gm food.				
per cent	gm.		gm.	gm.	gm.
3	0.54	1844 ♀ *	59.0	117.8	37.5
		1846 ♀ *	54.0	108.4	30.5
		1850 ♂ *	75.0	142.3	45.5
		2054 ♂ †	60.0	136.9	39.0
2	0.36	2268 ♂ †	69.0	150.2	35.0
		2271 ♂ †	65.5	148.7	35.5
1.5	0.27	2363 ♂ †	66.0	136.7	30.5
		2366 ♂ †	67.0	130.2	25.5
1	0.18	2291 ♂ †	64.5	136.6	21.5
		2294 ♂ †	61.5	113.7	9.5
None.	0.00	240 ♀ ‡	65.5	110.8	3.0
		249 ♀ §	65.5	87.4	6.0
		254 ♀ **	66.5	95.3	8.0
		1850 ♂ *	65.5	103.1	8.0

\* See Chart I, *J. Biol. Chem.*, 1914, xvii, 342.

† See Appendix, Chart IV.

‡ See Chart VII, *J. Biol. Chem.*, 1912, xii, 496.

§ See Chart VIII, *J. Biol. Chem.*, 1912, xii, 498.

\*\* See Chart IX, *J. Biol. Chem.*, 1912, xii, 499.

<sup>17</sup> Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325. (See Chart I, p. 342.)

Inasmuch as gliadin, which yields nearly 1 per cent of lysine, in addition to tryptophane, fails to promote growth until a supplement of 1 or more per cent of lysine is added to the food containing it, one can now readily understand why the zein food failed to promote growth satisfactorily, even with the addition of plenty of tryptophane, unless 2 per cent or more of lysine was furnished with the protein (see Charts II and III). Some growth may occur with smaller additions, but it is below the normal average and is limited by the proportion of the essential amino-acid present in smallest amount.

The application of some of these ideas regarding the indispensability of an adequate supply of certain amino-acids for growth has recently been attempted by Buckner, Nollau, and Kastle in the case of young chicks.<sup>18</sup> Their plan was to select mixtures of feeds which should be high and low, respectively, in lysine. The amount of this amino-acid was estimated by hydrolyzing the grain rations as a whole and determining the partition of nitrogen by the Van Slyke method. If the results of analyses made in this manner are trustworthy, the outcome of these feeding experiments is of much interest. They showed a very marked difference in the effects of the two grain mixtures, the rations of assumed low lysine content leading to decided retardation of growth. There are many variables besides that of the amino-acid yield in any attempt to compound food mixtures from ordinary feeds.

In a recent publication Abderhalden<sup>19</sup> has discussed what he designates as the "biologische Wertigkeit" of a number of the amino-acids obtained by the complete disintegration of protein with digestive enzymes. In several respects his results are comparable with studies which we have presented and repeatedly discussed in several communications in the past. His procedure has, in the main, consisted in feeding to dogs and rats mixtures of amino-acids obtained by digestion: and using the nitrogen balance as an evidence of nutritive equilibrium, he has ascertained the effect of such mixtures before and after the removal of individual amino-acids. In this way, quite in harmony with evidence which we have presented earlier, he has come to the conclusion that

<sup>18</sup> Buckner, G. D., Nollau, E. H., and Kastle, J. H., *Am. J. Physiol.*, 1915, xxxix, 162.

<sup>19</sup> Abderhalden, E., *Z. physiol. Chem.*, 1915, xcvi, 1.



tryptophane is an indispensable component of the dietary. Abderhalden's experiments with lysine, though pointing in the same direction, are far less conclusive.

#### SUMMARY.

Additional experiments are reported in this paper to show the part played by tryptophane and lysine in the metabolism of maintenance and growth. The quantity of these amino-acids available in the diet can be made the limiting factors which determine the nutritive equilibrium and possibilities for increment of size in an individual. They afford an important illustration of the "law of minimum" applied to essential nitrogenous components of the food supply.

## APPENDIX.

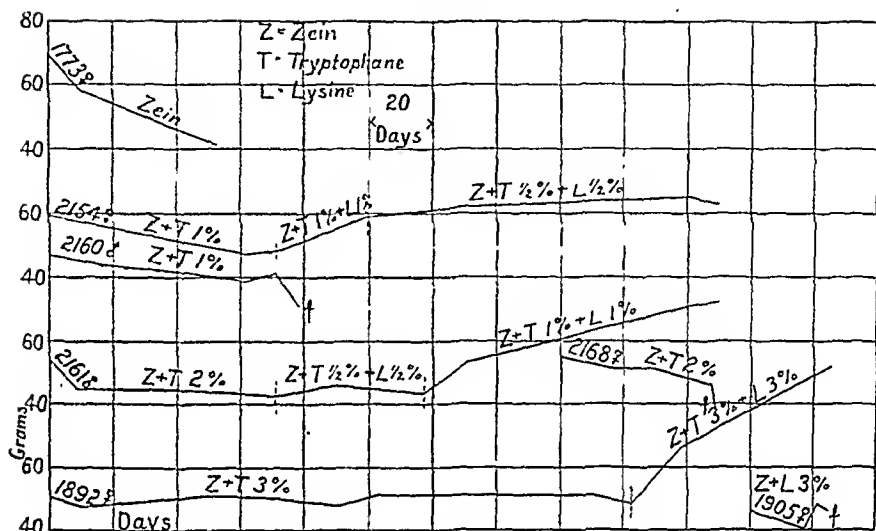


CHART I. Showing the effect of additions of varying proportions of tryptophane, lysine, or both, to zein foods. It will be noted that no increment of body weight takes place unless both of these amino-acids are added. The rate of growth appears to be limited by the proportions of these amino-acids furnished, thus illustrating the "law of minimum." Special attention is directed to the unique record of Rat 1892 described in detail in the text. The food, with which the lysine or tryptophane was incorporated, was composed essentially as follows:

	gm. .
Zein + amino-acid...	18
"Protein-free" milk.....	28
Starch.....	27
Butter fat.....	18
Lard.....	9
Water.....	15

In all cases the quantities of amino-acids used are expressed in percentages of the protein present—not in terms of the total food.

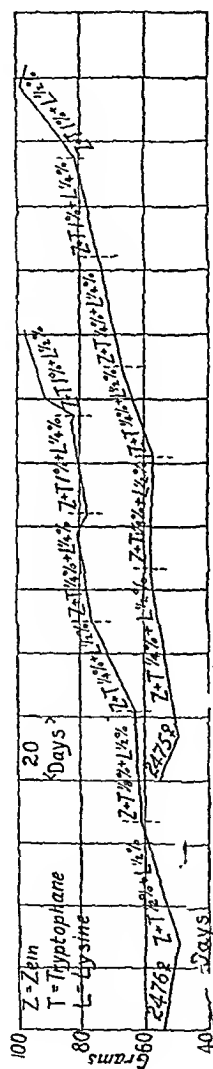


CHART II. Showing the influence of additions of small proportions of lysine and tryptophane upon the body weight of small rats. These should be compared with the records exhibited in Chart I, in connection with which the general composition of the foods used has been indicated.

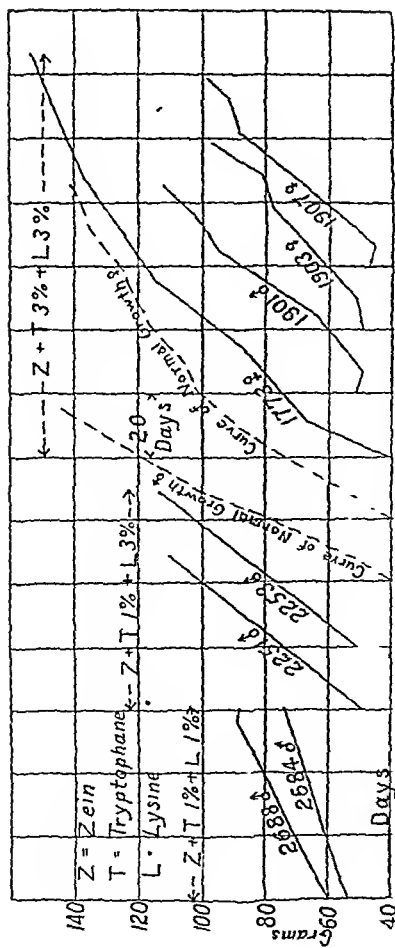
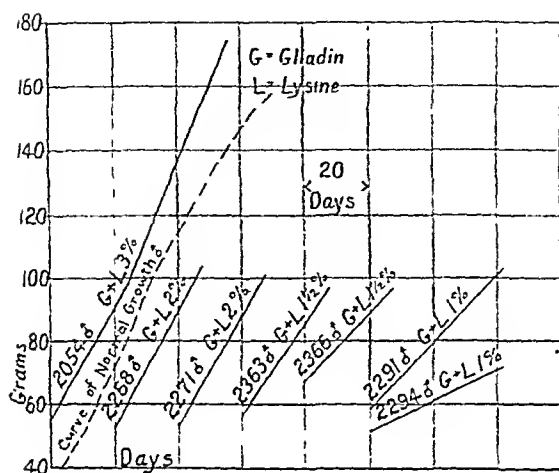


CHART III. Showing the proportions of tryptophane and lysine needed to promote good growth with zein foods. These records indicate clearly that to obtain maximal growth effects both amino-acids must be present in suitable quantities. The general composition of the foods used has been indicated in Chart I.



[CHART IV. Showing the necessity of adequate additions of lysine in order to produce satisfactory growth upon gliadin foods. In considering the total lysine content of the ration it should be recalled that the gliadin—of itself inadequate to produce a food suitable for growth—is estimated to yield nearly 1 per cent of lysine. The composition of the ration was essentially as follows:

	per cent
Gliadin and lysine.....	18
"Protein-free" milk.....	28
Starch.....	22
Butter fat ..	18
Lard.....	14

The added lysine replaced a part of the gliadin. Additional data regarding these animals are given in the table on p. 6.

# STUDIES IN THE SYNTHESIS OF HIPPURIC ACID IN THE ANIMAL ORGANISM.

## III. THE EXCRETION OF URIC ACID IN MAN AFTER INGESTION OF SODIUM BENZOATE.

BY HOWARD B. LEWIS AND WALTER G. KARR.

(From the Laboratory of Physiological Chemistry of the University of Illinois, Urbana.)

(Received for publication, March 15, 1916.)

Much confusion exists in the vast mass of experimental data accumulated in recent years concerning the influence of benzoic acid or its salts on the nitrogenous metabolism of man. With regard to uric acid excretion, the greater number of observers<sup>1</sup> assert that the ingestion of benzoic acid has no effect, or results in a slightly increased elimination. The therapeutic use of benzoates in gout and rheumatism, however, still continues, although they have been replaced to a large extent by atophan and salicylic acid derivatives. That salicylic acid, closely related to benzoic, when administered causes a marked increase in the uric acid excretion, has repeatedly been shown.<sup>2</sup> Recently Denis<sup>3</sup> has reported three cases in which the relations between the uric acid concentration of the blood and the urinary elimination of uric acid were studied before and after the ingestion of 8 gm. of sodium benzoate. As a result of these studies Denis concludes: "Benzoic acid like salicylic acid causes, when taken in large doses, a marked decrease in the uric acid content of the blood and an increase in the uric acid content of the urine. The effect, however, is not so constant or so marked as with the former drug." From a care-

<sup>1</sup> Compare Keller, W., *Ann. Chem.*, 1842, xliii, 108. Lewandowsky, M., *Z. klin. Med.*, 1900, xl, 202. Chittenden, R. H., Long, J. H., and Herter, C. A., *U. S. Dept. Agric., Report No. 88*, 1909, pp. 58, 586.

<sup>2</sup> Rockwood, E. W., *Am. J. Physiol.*, 1909-10, xxv, 34. Fine, M. S., and Chace, A. F., *J. Biol. Chem.*, 1915, xxi, 371. Denis, W., *J. Pharm. and Exp. Therap.*, 1915, vii, 255.

<sup>3</sup> Denis, W., *J. Pharm. and Exp. Therap.*, 1915, vii, 601.

ful study of the figures presented, the conclusions in so far as they concern the urine would seem to be open to question, as in only one of the three cases is the increase beyond normal variations.

In connection with former work by one of us<sup>4</sup> on the rate of elimination of hippuric acid after benzoate ingestion, the observation was made that the uric acid content of the urine was very low in the periods immediately following the ingestion of the benzoate, those periods in which the elimination of the greater part of the hippuric acid takes place. It seemed probable that any variations in the uric acid excretion due to the benzoate ingestion would be evident if the elimination was studied over short periods of time, coincident with the periods of maximal hippuric acid excretion, and that relations which would be obscured in the normal variations of 24 hour periods might be observed. With this in view the following series of experiments were undertaken.

The subjects of the experiments were healthy young men, students or instructors in the laboratory. For at least 2 days prior to the experimental periods as well as during the experiment the subjects were maintained on a purine-free diet. The experimental periods consisted of 3 or 4 days. The urine was collected at regular 2 hour intervals throughout the day with a 12 hour period at night. No attempt was made to maintain the diet quantitatively uniform throughout the experiments. Each subject, however, partook of a uniform breakfast daily. The benzoate was dissolved in water and administered with the breakfast. Subject A ate no food prior to the midday meal and received the benzoate at 8 a.m. Uric acid was determined by Benedict and Hitchcock's<sup>5</sup> modification of the colorimetric method of Folin and Denis, a method which in our experience gives values checking closely with those obtained by the older method of Folin and Shaffer provided only 0.5 mg. of uric acid is used in the standard solution and diluted to 50 cc. Creatinine was determined by the method of Folin and total benzoic acid by the method of Folin and Flanders.

In order to see whether the presence of large quantities of hippuric acid in small volumes of urine, a condition met with in the experimental days of benzoate ingestion, in any way affected

<sup>4</sup> Lewis, H. B., *J. Biol. Chem.*, 1914, xviii, 225.

<sup>5</sup> Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xv, 619.

the quantitative determination of uric acid by the method employed, the following experiments were carried out.

1. To 25 cc. of one of the experimental urines of a normal day 0.6 gm. sodium hippurate was added and the uric acid determined in 5 cc. in comparison with the original urine.

Original urine.	Urine after addition of sodium hippurate.
Colorimeter reading..... 20.8 mm.	21.8 mm. 22.0 " 21.7 " 21.8 " average of three determinations.
Uric acid calculated for total volume of period (400 cc.)..... 48 mg.	45.8 mg.

2. The same procedure was followed with another urine, 0.6 gm. sodium hippurate being added as before.

Original urine.	Urine after addition of sodium hippurate.
Colorimeter reading, average of three determinations..... 25.5 mm.	27.5 mm., average of four determinations.
Uric acid calculated for total volume of period (600 cc.)..... 59 mg.	55 mg.

Repetition of the above experiments with different urines gave results similarly low, from 3 to 4 mg. lower than the results for the urine without the addition of sodium hippurate. It was observed that in the urine with the addition of the hippurate, the precipitate was less flocculent and less bulky than was the case with the original urine. The precipitate resembled that obtained with pure uric acid solutions, rather than the mixed precipitate of phosphates and uric acid obtained normally with urines by this method. We believe that the smaller bulk of the precipitate resulted in a less compact mass in the bottom of the centrifuge tube, which caused a slight loss when the supernatant liquid was poured off. We were unable to obtain the typical bulky flocculent precipitate even when large amounts of the ammoniacal silver magnesium solution were added. The quantities of hippurate added in the above experi-



ments were much greater than the amounts present in any of the experimental urines, so that the figures obtained above represent the maximum possible error under the conditions, with which we dealt in the present experiments. Any changes in the uric acid content of the urine following benzoate ingestion must then be well above any possible error of the method in the presence of hippuric acid, in order to be significant.

A study of Table II shows that *coincident with the maximal elimination of the ingested benzoate (during the first 4 hours after administration, compare Table VI), the uric acid excretion is markedly diminished, a decrease of 50 to 70 per cent as compared with the excretion during the corresponding periods of the fore and after days.* These decreases while marked in the shorter periods are obscured in the figures for 24 hours, in which, although decreased elimination is evident, the variations are hardly more than normal fluctuations. This is especially marked in the experiments with Subjects A and L. When, however, the results for the first 4 hours of the day are summarized (Table I), the variations are seen to be well above any normal fluctuations or experimental error.

TABLE I

*Elimination of Uric Acid and Creatinine during the First 4 Hours of the Day.*

Subject	Day	Uric acid	Creatinine	Sodium benzoate administered
		mg	mg.	gm
H. B. L	Fore	140	311	0
	Fore .	150	305	0
	Benzoate	71	293	8
	After .	142	341	0
W. G. K	Fore	128	402	0
	Fore	122	313	0
	Benzoate	47	354	8
	After	152	365	0
L A .	Fore	89	290	0
	Benzoate	34	261	7
	After	118	299	0

Two possible explanations of this decrease suggest themselves. The changes might be due to a delayed elimination of the uric acid

by the kidneys, a retardation which should be followed by a compensatory increase in the later periods of the day. An inspection of the tables shows that no increased elimination in any way comparable to the decreases in the elimination of the earlier periods or greater than the normal variations of the fore and after days occurred. The possibility that these changes are the result of a general lowering of the plane of endogenous metabolism due to the toxic action of benzoic acid must also be taken into consideration. In order to test this hypothesis, creatinine was also determined throughout the experiments. No changes in the elimination of creatinine were observed (Tables I and IV). If the decreased excretion of uric acid observed is the result of a decreased formation of uric acid due to lowering of the level of nuclear metabolism under the influence of sodium benzoate, this must be considered as a specific influence on nuclear metabolism rather than on general endogenous metabolism, since the creatinine elimination remains normal. Whether the gastro-intestinal irritation commonly ascribed to the influence of large doses of benzoic acid would cause an increase in the endogenous uric acid metabolism is not clear from our present knowledge.<sup>6</sup> Certainly there is no evidence of the opposite effect.

The ingestion of benzoic acid results in the production of glyco-coll from sources as yet unknown, for purposes of detoxication. Is it not possible that uric acid may be broken down to yield glyco-coll or some other substance for detoxication, or may even itself be conjugated with benzoic acid? Uric acid is present normally in small amounts in human blood and tissues (1 to 2 mg. per 100 cc. of blood according to Folin and Denis<sup>7</sup>), while proteins and amino-acids, in the last analysis the sources of glyco-coll, occur in much larger amounts. If then, as has been suggested, uric acid contributes in some unknown way to the detoxication of benzoic acid, one would expect the reactions to proceed according to the masses of substances available, the greater rôle in the detoxication being played by the substance most abundant; *i.e.*, glyco-coll and its precursors to yield hippuric acid, and the less abundant uric acid playing a less important rôle in detoxication.

<sup>6</sup> Mendel, L. B., and Stehle, R. L., *J. Biol. Chem.*, 1915, xxii, 215.

<sup>7</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1913, xiv, 29.

In connection with this problem it seemed also of interest to determine whether this effect of benzoic acid on uric acid excretion was also produced by hippuric acid administered *per os*. Is the effect of the benzoic acid due to a toxic action on the cells, resulting in a decreased formation of uric acid, to the utilization of the uric acid formed for detoxication, or to the presence of hippuric acid circulating in the system? The experiments were conducted in the same manner as those previously described in this paper. An amount of sodium hippurate equivalent to the benzoate fed in the first series was administered.

As seen in Tables III and V, no significant changes in the elimination of creatinine or uric acid resulted from the administration of large doses of sodium hippurate. The decreased uric acid excretion observed after benzoate ingestion would seem to be due to the effect of the benzoic acid itself and not to its conjugated, non-toxic derivative, hippuric acid.

Notable also in the series of experiments is the slower elimination of the hippuric acid after the administration of sodium hippurate, than after benzoate administration (Table VI).

TABLE II.

*The Influence of Benzoate Ingestion on Uric Acid Excretion.*

Subject.	Day.	Sodium benzoate fed. gm.	Uric acid in mg. excreted for period ending						Total uric acid in 24 hrs.
			9 a. m.	11 a. m.	1 p. m.	3 p. m.	5 p. m.	7 p. m.	
H. B. L. Wt. 86 kg.	1	0	62	78	53	64	50	50	300
	2	0	75	75	64	65	56	34	210
	3	8	30	41	52	66	46	45	255
	4	0	78	64	62	74	43	Lost.	212
W. G. K. Wt. 74 kg.	1	0	55	73	58	45	52	53	239
	2	0	55	67	63	42	53	47	228
	3	8	28	19	43	32	36	39	259
	4	0	78	74	63	61	53	48	166
L. A.* Wt. 59.1 kg.	1	0	55	34	32	36	39	47	212
	2	7	16	18	49	37	40	54	206
	3	0	57	61	46	47	50	52	203

\* Collection of urine beginning at 8 a.m. in 2 hr. periods.

TABLE III.

*The Influence of Hippurate Ingestion on Uric Acid Excretion.*

Subject.	Day.	Sodium hip- purate fed.	Uric acid in mg. excreted for period ending						Total uric acid in 24 hrs
			9 a. m.	11 a. m.	1 p. m.	3 p. m.	5 p. m.	7 p. m.	7 a. m.
		<i>gm.</i>							<i>mg.</i>
H. B. L.	1	0	89	70	63	68	45	40	285
Wt.	2	11.2	65	58	46	69	65	Lost.	305
86 kg.	3	0	72	68	48	43	46	32	216
W. G. K.	1	0	65	67	49	48	44	31	239
Wt.	2	11.2	60	81	51	76	54	41	246
74 kg.	3	0	78	66	42	47	31	37	226

TABLE IV

*The Influence of Benzoate Ingestion on Creatinine Excretion.*

Subject.	Day.	Sodium ben- zoate fed.	Creatinine in mg. excreted for period ending						Total creati- nine in 24 hrs
			9 a. m.	11 a. m.	1 p. m.	3 p. m.	5 p. m.	7 p. m.	7 a. m.
		<i>gm.</i>							<i>mg.</i>
H. B. L.	1	0	149	162	143	162	126	144	810
Wt.	2	0	168	137	155	143	158	116	810
86 kg.	3	8	163	130	144	162	137	117	730
	4	0	205	136	148	158	135	Lost.	675
W. G. K.	1	0	189	213	182	171	197	171	1088
Wt.	2	0	176	137	164	176	247	187	894
74 kg.	3	8	193	161	174	202	127	154	988
	4	0	186	179	194	177	177	178	868
L. A.*	1	0	162	128	125	147	180	142	810
Wt.	2	7	140	121	162	127	174	150	765
59.1 kg.	3	0	152	147	142	130	165	147	837

\* Collection of urine beginning at 8 a.m. in 2 hr. periods.

TABLE V.

*The Influence of Hippurate Ingestion on Creatinine Excretion.*

Subject	Day	Sodium hip- purate fed	Creatinine in mg excreted for period ending						Total creati- nine in 24 hrs	
			9 a m	11 a m	1 p m.	3 p m.	5 p m	7 p m		7 a m.
		gm							mg	
H. B. L	1	0	248	169	169	179	145	168	965	2043
Wt	2	11 2	162	162	162	173	169	Lost.	980	
86 kg	3	0	156	152	159	147	170	202	1039	2025
W. G. K.	1	0	197	195	202	196	202	152	1100	2244
Wt	2	11 2	193	187	170	225	206	188	1048	2217
74 kg	3	0	219	208	190	221	170	163	1318	2489

TABLE VI

*Total Benzoic Acid Excretion following Ingestion of Sodium Benzoate or Sodium Hippurate.*

Subject	Day	Sodium benzoate or sodium hippurate fed calculated as benzoic acid	Total benzoic acid in gm excreted during period ending				Total benzoic acid in 8 hrs
			9 a m	11 a m	1 p m	3 p m	
		gm					gm
H. B. L	3	Benzoate. 6 76	2 94	2 86	0 45	0 16	6 41
H. B. L	2	Hippurate. 6 76	2 56	1 91	0 54	0 51	5 52
W. G. K	3	Benzoate. 6 76	1 96	2 57	1 48	0 21	6 22
W. G. K	2	Hippurate. 6 76	1 09	2 80	1 34	0 72	5 95
L. A.*	2	Benzoate. 5 92	1 95	2 39	1 39	0 14	5 87

\* Collection of urine beginning at 8 a.m. in 2 hr. periods.

# STUDIES IN THE BLOOD RELATIONSHIP OF ANIMALS AS DISPLAYED IN THE COMPOSITIONS OF THE SERUM PROTEINS.

## V. THE PERCENTAGE OF NON-PROTEINS IN THE SERA OF CERTAIN ANIMALS AND BIRDS.

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California, Berkeley.)

(Received for publication, March 13, 1916.)

This investigation was undertaken with a view to correcting and extending the figures given in former articles of this series by adding the determination of non-proteins for each animal.

In former articles of this series<sup>1</sup> it was assumed that the amount of non-proteins in the blood sera of all animals was about the same; but it has since been ascertained that this assumption is not accurate;<sup>2</sup> and the present work was undertaken to ascertain the amount of non-protein in the blood of each kind of animal formerly studied and to correct the figures which are affected by the revised estimation of the non-proteins. In the present investigation Robertson's refractometric method<sup>2</sup> was used in so far as the determination of non-proteins is concerned, and in cases where the concentration of the serum being examined was much greater or less than that examined by former investigators, the ascertained amount of non-protein was increased or reduced in like proportion before correcting the previously published figures.

The following table shows the refractive indices obtained, the percentages of albumin, globulin, and total proteins determined by previous investigators, and the figures as corrected by the present investigation. Where the percentage of non-protein in

<sup>1</sup> Robertson, T. B., *J. Biol. Chem.*, 1912, xi, 179; 1912-13, xiii, 325. Woolsey, J. H., *ibid.*, 1913, xiv, 433. Thompson, W. B., *ibid.*, 1915, xx, 1. Briggs, R. S., *ibid.*, 1915, xx, 7.

<sup>2</sup> Robertson, *J. Biol. Chem.*, 1915, xxii, 233.

hydrolyzed in solution, and the consequence is the setting free of soluble acid; that is, the presence of excess of acid in the soil. Of course the acid radicles combine with the stronger bases, such as calcium and magnesium, but the acid condition of any soil is due to the fact that it does not have a sufficient supply of the strong bases; hence in many soils aluminium and iron necessarily supply the basic radicle. It thus happens that in many cases the apparent acidity of the extract from the soil as determined by titration with standard alkali in the presence of phenolphthalein, is found to be proportional to the amount of aluminium salts present in the solution, and evidently represents the amount of alkali required to precipitate the aluminium rather than actual free acidity. Accordingly, the addition of strong bases as calcium to the soil or treatment such as ignition which makes soil constituents less reactive, will stop or decrease the production of acid by the application of soluble salts to the soils.

From the view of soil acidity above stated, it appeared to be of interest to investigate the toxicity of soluble aluminium and iron salts and its relation to their acidity. As the soluble iron salts are generally present in negligible traces in the soil solution, we will confine our investigation to aluminium salts.

There are a few publications on the physiological action of aluminium salts on the growth of plants. Miecheels and De Heen investigated the effect of aluminium salts on the germination of wheat, with the result that, while they found alumina or kaolin to be beneficial, the addition of soluble aluminium salts proved injurious. House and Gies<sup>3</sup> found that the toxicity of aluminium salts is dependent entirely upon the concentration. Yamano<sup>4</sup> ascertained by pot experiment on barley and flax that moderate amounts of aluminium salts have a stimulating action on plant development. In water cultures, however, 0.2 per cent of alum acted injuriously after 3 weeks, while 0.8 per cent killed the plant in a few days. Hébert<sup>5</sup> studied the toxicity of chromium and aluminium sulfates in comparison with the sulfates of some of the rare metals. Progressively increasing amounts of these compounds were tested on germinated seeds of pea, wheat, and rape. Aluminium, chromium, and some of the rare metal sulfates were found to be strongly poisonous. Duggar<sup>6</sup> reports aluminium

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<sup>2</sup> Miecheels, H., and De Heen, P., *Bull. Acad. roy. Belg.*, 1905, 520.

<sup>3</sup> House, H. D., and Gies, W. J., *Am. J. Physiol.* 1905-06, xv, p. xix.

<sup>4</sup> Yamano, Y., *Bull. College Agric., Tokyo Imperial Univ.*, 1905, vi, 429.

<sup>5</sup> Hébert, A., *Bull. Soc. chim.*, 1907, series 4, i, 1026.

<sup>6</sup> Duggar, B. M., *Plant Physiology*, New York, 1911.

chloride and sulfate extremely toxic to pea grown in water cultures. Abbott, Conner, and Smalley<sup>7</sup> found that very dilute solutions of aluminium nitrate are toxic to corn seedlings in water culture in the presence of mineral nutrients. The toxicity of aluminium nitrate was found to be approximately equal to that of nitric acid of the same normality. E. Kratzmann<sup>8</sup> proved that the growth of higher plants is retarded by the presence of 0.05 per cent of aluminium salts, but very small amounts (0.0001 per cent) have a slight stimulating action. Ruprecht<sup>9</sup> found also that aluminium sulfate, when present in culture solutions in concentrations of aluminium greater than forty parts per million, has a very toxic action on clover seedlings.

From the above review it will be seen that, although there is evidence that aluminium salts are toxic, there has been no research work done on the question of the relationship of the toxicity of aluminium salts to their acidity, except the article by Abbott, Conner, and Smalley which touches on this matter.

Water cultures were employed in the investigation. Though of limited value in soil fertility investigation from the standpoint of agriculture, this method of investigation furnishes an excellent means of studying special phases in which physiological effects are important. For the solution I used aluminium chloride in concentrations of  $\frac{N}{1,000}$  to  $\frac{N}{20,000}$  as will be seen in Table I, and hydrochloric acid solutions of same concentrations were used for comparison. In the experiment I started with the young rice seedlings, 10 mm. high, which were grown in distilled water from seeds of almost uniform size and specific gravity. Fourteen beakers about 5.5 cm. in diameter and 7 cm. deep, each containing 30 cc. of solution, were used for the experiment, the seedlings being placed in the solution, while one beaker containing distilled water served as a control. Five seedlings were grown in each culture at ordinary temperature and the evaporated water was supplemented with distilled water from time to time to keep the solutions always at their initial dilutions. After 13 days, the difference in development was striking, when the following measurements were made.

<sup>7</sup> Abbott, J. B., Conner, S. D., and Smalley, H. R., *Ind. Experiment Station, Bull. 170*, 1913, 329.

<sup>8</sup> Kratzmann, E., *Chem. Ztg.*, 1914, xxxviii, 1040.

<sup>9</sup> Ruprecht, R. W., *Mass. Experiment Station, Bull. 161*, 1915, 125.



TABLE I.

		Concentration.							
		$\frac{N}{1,000}$	$\frac{N}{2,500}$	$\frac{N}{5,000}$	$\frac{N}{7,500}$	$\frac{N}{10,000}$	$\frac{N}{15,000}$	$\frac{N}{20,000}$	Control.
AlCl <sub>3</sub>	{ Length of leaf, mm	50	51	51	55	57	57	57	57
	{ Length of root, mm	12	12	16	26	36	36	52	52
	{ No. of roots	5*	8*	8*	8	7	7	7	7
HCl	{ Length of leaf, mm	41	48	48	55	57	57	62	57
	{ Length of root, mm	26	33	33	56	55	52	52	52
	{ No. of roots	1	2*	7*	7	7	7	7	7

\* Only the primary root was well developed

Though a little difference of growth between the aluminium chloride and hydrochloric acid solutions was observed, the table shows that each solution in the same normality causes almost similar retardation as stated by Abbott, Conner, and Smalley, and concentrations greater than  $\frac{N}{7,500}$  are injurious to the growth of rice seedlings. From this result we cannot immediately conclude that the toxic action of aluminium chloride is due to the hydrogen ions formed by the hydrolysis of the salt in solution unless we have a reliable measurement of the hydrogen ion concentration in aluminium chloride solutions, because, though aluminium chloride is hydrolyzed in solution, we cannot consider that the hydrolysis is complete and that the concentration of hydrogen ion is the same as that in a solution of hydrochloric acid of the same normality.

An examination of the literature failed to reveal any data on the degree of hydrolysis or concentration of hydrogen ions of aluminium chloride solutions. I have, therefore, measured the concentration of hydrogen ions in some of the above mentioned solutions of aluminium chloride by the method of the gas chain. Details of the apparatus and method of manipulation are now too well known to require description. For the determination I had the assistance and apparatus of Mr. C. L. A. Schmidt, to whom I express my thanks. The concentration of hydrogen ions was measured in the solution of  $\frac{N}{7,500}$  aluminium chloride, which seems to be a critical concentration of toxicity, and in two other dilutions, that is,  $\frac{N}{1,000}$  and  $\frac{N}{5,000}$ , which are toxic to the growth. The

TABLE 1.

		Concentration.							
		$\frac{N}{1,000}$	$\frac{N}{2,500}$	$\frac{N}{5,000}$	$\frac{N}{7,500}$	$\frac{N}{10,000}$	$\frac{N}{15,000}$	$\frac{N}{20,000}$	Control.
AlCl <sub>3</sub>	Length of leaf, mm	50	51	51	55	57	57	57	57
	Length of root, mm	12	12	16	26	36	36	52	52
	No. of roots	5*	8*	8*	8	7	7	7	7
HCl	Length of leaf, mm	41	48	48	55	57	57	62	57
	Length of root, mm	26	33	33	56	55	52	52	52
	No. of roots	1	2*	7*	7	7	7	7	7

\* Only the primary root was well developed

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result obtained is as follows. The concentration of hydrogen ion of hydrochloric acid in same normality was also calculated under the supposition that the hydrochloric acid is completely dissociated into hydrogen and chlorine ions in such dilutions.

TABLE II.

	Concentration.		
	$\frac{N}{1,000}$	$\frac{N}{5,000}$	$\frac{N}{7,500}$
HCl.....	$1,000 \times 10^{-3}$	$2,000 \times 10^{-4}$	$1,330 \times 10^{-4}$
AlCl <sub>3</sub> .....	$0,317 \times 10^{-3}$	$0,716 \times 10^{-4}$	$0,505 \times 10^{-4}$
HCl: AlCl <sub>3</sub> .....	1:0,317	1:0,358	1:0,379

Though the results obtained by the determination are not very accurate, because the measurement was very difficult owing to the high internal resistance of such solutions, still the great differences between the hydrogen ion concentration in the aluminium chloride and hydrochloric acid solutions could not be attributed to the experimental error. From this result it may therefore be safely concluded that the actual acidity of aluminium chloride solutions is less than that of hydrochloric acid. The toxicity of aluminium chloride, as we have seen, however, appears to be almost exactly the same as that of hydrochloric acid of the same normality. It is, therefore, clear that the acidity of aluminium chloride solutions is not the cause of the toxic action of the salt. The toxic effect of aluminium chloride solution must be due to other factors.

## SUMMARY.

Taking the above results into consideration we may safely formulate the following conclusions:

1. Aluminium chloride is toxic to the growth of rice seedlings even in dilute solution.

2. The toxic effect of aluminium chloride upon the growth of rice seedlings appears in concentrations greater than  $\frac{N}{7,500}$ .

3. The toxicity of aluminium chloride seems to be approximately equal to that of hydrochloric acid of the same normality.

4. The toxic action of aluminium chloride is not due to the hydrogen ion formed by hydrolysis of the salt in solution.

5. The concentration of hydrogen ions formed by the hydrolysis of aluminium chloride is less than that formed by dissociation of hydrochloric acid of the same normality.

6. Since the chlorine ion is not toxic to the growth of rice seedlings in such dilute solution,<sup>10</sup> colloidal aluminium hydroxide or unhydrolyzed aluminium chloride molecules or aluminium ions may be the toxic factors.

7. The toxicity of soluble aluminium salts is dependent upon the amount of aluminium itself. The determination of soil acidity by titration in which the soil extract is titrated with standard alkali is a logical method of determining the amount of bases which should be added to the soil for the amelioration of its infertility; because, although the titration does not indicate the true acidity of the soil, yet it does afford a measure of the bases which must be added to neutralize the free acid and decompose the aluminium salts, either or both of which may be responsible for the infertility.

In conclusion, the author wishes to express his thanks to Dr. T. Brailsford Robertson for his kind advice during this experiment.

<sup>10</sup> Miyake, K., *Tr. Sapporo Natural History Soc.*, Japan, 1914, v, 91.

# SOLUBILITY DATA FOR VARIOUS SALTS OF LAURIC, MYRISTIC, PALMITIC, AND STEARIC ACIDS.\*

By C. A. JACOBSON AND AUGUST HOLMES.

(From the Department of Chemistry, University of Nevada, Reno.)

(Received for publication, March 15, 1916.)

The isolation of individual members of the higher saturated fatty acids, when occurring in mixtures of such acids, has always been fraught with considerable, if not insurmountable difficulties.

The authors, in their characterization of the constituents of alfalfa seed oil,<sup>1</sup> were confronted with the same difficulties. After applying all the known methods that were available for these separations, and finding that unsatisfactory results were obtained in every case when artificially prepared mixtures of the fatty acids were employed, it was decided to investigate the solubilities of various salts of lauric, myristic, palmitic, and stearic acids in a number of organic solvents as well as in water.

It was hoped that the solubility data thus obtained would furnish the means for a scheme of separation of the different acids, but just how far these anticipations were realized will be discussed in another paper.

The property of solubility has furnished the basis for more systems of separation of chemical substances than any other, although differences of melting point, boiling point, and volatilization in steam are properties upon which schemes of separation have been founded.

The following tables include the solubility of the lithium, magnesium, beryllium, barium, lead, and silver salts of lauric, myristic, palmitic, and stearic acids in two or more of the following solvents: water, ethyl and methyl alcohol, ether, benzene, ethyl acetate, methyl acetate, amyl alcohol, amyl acetate, chloro-

\* This investigation was carried out at the Nevada Agricultural Experiment Station with funds obtained under the Adams Act.

<sup>1</sup> Jacobson, C. A., and Holmes, A., *J. Am. Chem. Soc.*, 1916, xxxviii, 480.

form, and acetone, at room temperature, 25°, 35°, and 50°, whenever the boiling point of the solvent permitted.

The solvents were selected according to their most probable applicability along this line. They were of the highest purity obtainable and always redistilled whenever any question as to their purity arose. In general the salts of the fatty acids were made from the acetates of the metals, but a detailed description of the preparation of each salt will be given in connection with its table of solubilities.

An excess of the salt to be investigated was put into a 100 cc. round bottom flask having a neck about 5 inches long. Four such flasks were used at the same time for the four different salts of a given metal and the flasks filled nearly full with the solvent to be employed. The flasks were then stoppered and clamped in a shaking device, which was so arranged that the flasks, excepting the upper part of the necks, were immersed in a bath of water whose temperature was kept constant to within 0.5°. An electric motor was used for shaking the flasks in the water bath and the agitation continued for 2 hours after the liquid in the flasks had assumed the temperature of the water in the bath. The shaking was then interrupted and the suspended matter allowed to settle, after which about 10 cc. of the solution were rapidly drawn off with a carefully calibrated pipette, the solution was weighed in a covered weighing tube, and finally the solvent evaporated off. From the weight of the residue in the weighing tubes the solubility was calculated in terms of gm. of salt, soluble in 100 gm. of solvent.

We did not attempt to obtain absolute solubilities in any case, although most of the data here submitted will approach the absolute values very closely. It has been learned that in some instances a 6 hour shaking in contact with the solvent is not sufficient to secure maximum solubility, but for the solvents used the difference between the solubility after 2 and 6 hours' shaking is so slight that for all practical purposes it may be neglected. Amyl alcohol may be considered an exception to this rule for it was found that a 2 hour shaking in most cases did not suffice to produce a saturated solution. The results recorded under this solvent are those obtained after 2 hours' shaking, making them comparable with those of the other salts.

In the first table will be found the results of analyses of the four fatty acids and their salts which were employed in obtaining the following solubility data.

TABLE I.

	Lauric acid.	Myristic acid.	Palmitic acid.	Stearic acid.
M. p. found.	13 0°	19 7°	61 4°	69 0°
M. p. given....	13 6°	53 8°	62 0°	69.2°
Neutralization value found.....	280 0	228 5	220 4	199.3
Neutralization value calculated.....	280 5	216 1	219.1	197.5
M. p. Li salt.....	229 2-229 8°	223 6-221 2°	224-225°	220.5-221.5°
Per cent of Li in Li salt found.....	3 41	2 91	2 58	2.38
Per cent of Li calculated.....	3.48	2 99	2 66	2.41
M. p. Mg salt.....	150.4°	131 6°	121-122°	132°
Per cent of Mg in Mg salt found.....	5.73	5 09	4.51	4.20
Per cent of Mg calculated.....	5.75	5 08	4.54	4.11
M. p. Pb salt.....	101.6-104 8°	108.6-108 8°	112.2-112 4°	115.6-115.8°
Per cent of Pb in Pb salt found.....	33 66	31.01	28 24	26.50
Per cent of Pb calculated.....	31 28	31.31	28.86	26.77
Per cent of Ba in Ba salt found.....	25 82	24.20	21.62	19.60
Per cent of Ba calculated.....	25.64	23.22	21.20	19.52
M. p. Ag salt....	212-213°	211°	209°	205°
Per cent of Ag in Ag salt found....	34 78	32 45	29.74	28.05
Per cent of Ag calculated..	35 12	32 20	29.72	27.58

In Table II will be found the analyses of the beryllium salts of the four fatty acids which were prepared in the following manner: To 10 gm. of the acid dissolved in 95 per cent alcohol (the solution neutralized with alcoholic ammonium hydroxide, using litmus as the indicator), a calculated amount of beryllium nitrate dissolved in alcohol was added. The resulting precipitate was

washed several times with boiling alcohol, both by decantation and in a filter, then dried and subjected to analysis with the following results.

TABLE II

	Be laurate	Be myristate	Be palmitate	Be stearate
Per cent of Be found	3 71	3 48	3.08	2 81
Calculated for Be (Ac) <sub>2</sub>	2 23	1 96	1.75	1 58
Ratio $\frac{\text{Found}}{\text{Calculated}}$	1 66	1 77	1 76	1 78
Hence, calculated for Be(OH)Ac	4 04	3 59	3 23	2 87
Per cent of C found	63 85	66 19	67 95	70 17
Calculated for Be(OH)Ac	63 91	66 33	68 23	69 81
Per cent of H found	10 93	10 90	11 53	11 91
Calculated for Be(OH)Ac	10 74	11 13	11 48	11 73

The above results show that the basic salts of beryllium with the formula Be(OH)Ac were obtained rather than the normal Be(Ac)<sub>2</sub> salts. They were found to be only very slightly soluble in the general organic solvents, but for the sake of completeness their solubilities were determined in ethyl and methyl alcohol at 25°. Table III contains these data.

TABLE III

*Solubility of Acid Salts of Beryllium at 25°.*

	Laurate	Myristate	Palmitate	Stearate
Ethyl alcohol $\frac{\text{gm. salt}}{100 \text{ gm. solvent}}$	0 004	0 004	0 004	..
Methyl alcohol $\frac{\text{gm. salt}}{100 \text{ gm. solvent}}$	0 050	0 047	0 042	0 040

Table IV contains the solubility data for the lithium salts prepared by adding a calculated amount of lithium acetate to the alcoholic solutions of the respective fatty acids. The precipi-



tates formed were dissolved in boiling alcohol and the solutions allowed to stand over night in a cool place. The salts that had separated were washed and dried.

TABLE IV

*Solubility of Lanthanum Salts in Ethyl Alcohol (Absolute).*

	Salt	Solution	Solvent.	Salt in 100 gm solvent.
Temperature 20°				
	g	gm	gm	gm
Laurate..	0.0313	7.79	7.76	0.403
Myristate	0.0117	7.57	7.55	0.194
Palmitate	0.0075	7.84	7.80	0.096
Stearate	0.0056	7.71	7.73	0.072
Temperature 25.1°				
Laurate..	0.0312	7.69	7.66	0.447
Myristate..	0.0174	7.77	7.76	0.224
Palmitate	0.0092	7.80	7.79	0.118
Stearate..	0.0060	7.78	7.78	*0.089
Temperature 35°				
Laurate...	0.0118	7.69	7.65	0.546
Myristate.	0.0215	7.74	7.72	0.278
Palmitate.	0.0110	7.77	7.76	0.142
Stearate..	0.0082	7.73	7.72	0.106
Temperature 50°				
Laurate.	0.0594	7.66	7.60	0.782
Myristate	0.0335	7.69	7.66	0.435
Palmitate	0.0190	7.64	7.61	0.248
Stearate.	0.0154	7.69	7.67	0.200
Temperature 65°				
Laurate.	0.0827	7.28	7.20	1.149
Myristate	0.0490	7.38	7.33	0.669
Palmitate	0.0306	7.85	7.82	0.391
Stearate	0.0256	7.72	7.69	0.333

The solubilities here recorded are practically the absolute solubilities of the salts used, if the results of Partheil and Férié<sup>2</sup> can be taken to represent absolute solubilities.

TABLE V  
*Solubility of Lithium Salts in Methyl Alcohol*

	Salt	Solution	Solvent	Salt in 100 gm solvent
Temperature 15.2°				
	gm	gm	gm	gm.
Laurate	0.2442	7.97	7.73	3.159
Myristate	0.1055	7.95	7.84	1.346
Palmitate	0.0486	7.94	7.89	0.616
Stearate	0.0321	9.23	9.20	0.349
Temperature 25°				
Laurate	0.2883	7.93	7.64	3.773
Myristate	0.1299	7.86	7.73	1.680
Palmitate	0.0604	7.89	7.83	0.771
Stearate	0.0344	7.85	7.82	0.439
Temperature 34.6°				
Laurate	0.3463	7.88	7.53	4.597
Myristate	0.1684	7.85	7.68	2.193
Palmitate	0.0850	7.82	7.83	1.086
Stearate	0.0513	7.84	7.79	0.658
Temperature 50°				
Laurate	0.4487	7.82	7.37	6.088
Myristate	0.2329	7.49	7.25	3.281
Palmitate	0.1252	7.65	7.52	1.652
Stearate	0.0810	7.76	7.68	1.057

<sup>2</sup> Partheil, A. and Férié, P., *Arch. Pharm.*, 1903, ccxli, 545

TABLE VI.  
*Solubility of Lithium Salts in Water.*

	Salt	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 16.3°				
	gm.	gm.	gm.	gm.
Laurate .....	0 0152	9 86	9 84	0 154
Myristate.....	0 0027	9 85	9 85	0 027
Palmitate....	0 0010	9 84	9 84	0 010
Stearate...	0 0009	9 86	9 86	0 009
Temperature 25°				
Laurate.....	0 0184	9 86	9 84	0 187
Myristate....	0 0039	10 83	10 83	0 036
Palmitate....	0 0015	9 85	9 85	0 015
Stearate...	0 0010	9 83	9 83	0 010
Temperature 35°				
Laurate...	0 0203	9 83	9 81	0 207
Myristate .	0 0012	10 01	10 02	0 042
Palmitate...	0 0015	9 83	9 82	0 015
Stearate..	0 0010	9 12	9 12	0 010
Temperature 50°				
Laurate...	0 0274	9 84	9 78	0 280
Myristate	0 0064	9 79	9 79	0 062
Palmitate .				
Stearate .				

TABLE VII  
*Solubility of Lithium Salts in Ether.*

	Salt	Solution	Solvent.	Salt in 100 gm. solvent
Temperature 15.8°				
	gm.	gm.	gm.	gm.
Laurate...	0 0008	7 20	7 20	0 011
Myristate	0 0009	7 00	7 00	0 013
Palmitate	0 0005	7 09	7 09	0 007
Stearate .	0 0008	7 23	7 23	0 011
Temperature 25°				
Laurate .	0 0005	7 98	7 98	0 006
Myristate	0 0003	7 73	7 73	0 004
Palmitate	0 0006	8 35	8 35	0 007
Stearate.	0 0008	7 01	7 01	0 011

TABLE VIII

*Solubility of Lithium Salts in Amyl Alcohol.*

	Salt.	Solution	Solvent	Salt in 100 gm solvent.
Temperature 16°				
	gm	gm	gm.	gm
Laurate	0 0058	7 92	7 92	0 073
Myristate	0 0024	8 21	8 21	0 029
Palmitate	0 0016	8 33	8 32	0 019
Stearate	0 0008	7 45	7 45	0 011
Temperature 25 7°				
Laurate	0 0081	7 29	7 28	0 111
Myristate	0 0036	7 74	7 74	0 046
Palmitate	0 0024	7 48	7 47	0 032
Stearate	0 0022	7 88	7 88	0 028
Temperature 35°				
Laurate	0 0101	8 03	8 02	0 126
Myristate	0 0052	8 33	8 33	0 062
Palmitate	0 0028	8 37	8 37	0 033
Stearate	0 0024	7 86	7 86	0 031
Temperature 49 2°				
Laurate	0 0173	8 54	8 53	0 203
Myristate	0 0079	7 22	7 21	0 109
Palmitate	0 0052	7 49	7 48	0 069
Stearate	0 0046	7 61	7 61	0 060

TABLE IX

*Solubility of Lithium Salts in Chloroform*

	Salt	Solution	Solvent	Salt in 100 gm solvent
Temperature 15 2°				
	gm	gm	gm	gm
Laurate	0 0010	16 59	16 59	0 006
Myristate	0 0007	16 03	16 03	0 004
Palmitate	0 0006	15 60	15 60	0 004
Stearate	0 0007	16 37	16 37	0 004

TABLE X.  
*Solubility of Lithium Salts in Amyl Acetate.*

	Salt.	Solution.	Solvent.	Salt in 100 gm solvent.
Temperature 11.5°				
	gm	gm	gm.	gm.
Laurate .....	0 0056	8 23	8 23	0 068
Myristate.....	0 0031	8 10	8 10	0.037
Palmitate.....	0 0023	6 07	6 07	0 038
Stearate.....	0 0028	8.13	8 13	0 031
Temperature 25°				
	gm	gm	gm.	gm.
Laurate.....	0 0052	8 07	8 07	0 064
Myristate.....	0 0028	8 31	8 31	0.034
Palmitate.....	0 0020	8 26	8 26	0 024
Stearate....	0 0021	8 10	8 40	0 029
Temperature 35°				
	gm	gm	gm.	gm.
Laurate.....	0 0049	8 02	8 02	0 061
Myristate.....	0 0037	8 42	8 12	0 041
Palmitate.....	0 0031	8 44	8 43	0 037
Stearate.....	0 0021	7 81	7 81	0 031
Temperature 50°				
	gm	gm	gm.	gm.
Laurate.....	0 0052	8 58	8 57	0 061
Myristate ..	0 0038	8 52	8 52	0 015
Palmitate. ...	0 0030	8 33	8 32	0 036
Stearate.....	0 0040	8 97	8 97	0 044

TABLE XI.  
*Solubility of Lithium Salts in Methyl Acetate.*

	Salt	Solution	Solvent.	Salt in 100 gm. solvent.
Temperature 24.5°				
	gm.	gm.	gm.	gm.
Laurate..	0 0027	10 22	10 22	0 026
Myristate	0 0012	9 51	9 51	0 013
Palmitate..	0 0015	9 87	9 87	0 015
Stearate.....	0 0012	9 69	9 69	0 012

TABLE XII.

*Solubility of Lithium Salts in Acetone.*

	Salt.	Solution.	Solvent.	Salt in 100 gm solvent.
Temperature 15°				
	gm	gm.	gm.	gm.
Laurate .	0 0234	7 83	7.81	0 300
Myristate	0 0322	7 82	7 79	0 413
Palmitate .	0.0338	7 82	7.79	0 434
Stearate .	0.0437	7.70	7.76	0 571
Temperature 25°				
Laurate .	0 0293	7 82	7.79	0 376
Myristate	0 0305	6 86	6 83	0 447
Palmitate	0 0396	7 83	7.79	0 508
Stearate	0 0510	7 27	7.22	0 706
Temperature 35°				
Laurate	0 0329	7 67	7.64	0.430
Myristate	0 0385	7 71	7 67	0.502
Palmitate	0 0414	7 75	7.71	0 537
Stearate .	0 0509	7 73	7.68	0 663

The magnesium salts were prepared by adding a slight excess of magnesium acetate to the warm alcoholic solutions of the fatty acids and the solution was set aside to cool. The laurate and myristate were recrystallized from hot alcohol while the palmitate and stearate were repeatedly washed with alcohol and dried.

TABLE XIII.  
*Solubility of Magnesium Salts in Water.*

	Salt	Solution	Solvent.	Salt in 100 gm. solvent.
Temperature 15°				
	gm.	gm.	gm.	gm.
Laurate.....	0 0010	9 85	9 85	0.010
Myristate.....	0 0006	9 87	9.87	0.006
Palmitate.....	0 0005	9 81	9 84	0.005
Stearate. . . . .	0 0003	9 85	9 85	0 003
Temperature 25°				
Laurate.....	0 0007	9 85	9 85	0 007
Myristate ...	0 0006	9 73	9 73	0.006
Palmitate.....	0 0008	9 84	9 84	0.008
Stearate.. .	0 0004	9 86	9 86	0 004
Temperature 35°				
Laurate.... .	0 0010	9 83	9 83	0.010
Myristate.....	0 0007	9 83	9 83	0 007
Palmitate.....	0 0006	9 83	9.83	0.006
Stearate. . . . .	0 0007	9 80	9 80	0.007
Temperature 50°				
Laurate.....	0 0026	9 81	9 81	0 026
Myristate....	0 0014	9 79	9 79	0 014
Palmitate. . . .	0 0009	9 82	9 82	0.009
Stearate.. .	0 0008	9 79	9 79	0.008

TABLE XIV.

*Solubility of Magnesium Salts in Ethyl Alcohol (Absolute).*

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15°				
	gm.	gm.	gm.	gm.
Laurate.....	0.0404	7.82	7.78	0.519
Myristate.....	0.0123	7.79	7.78	0.158
Palmitate.....	0.0028	7.83	7.83	0.034
Stearate.....	0.0013	7.81	7.81	0.017
Temperature 25°				
	gm.	gm.	gm.	gm.
Laurate.....	0.0459	7.81	7.76	0.591
Myristate.....	0.0183	7.76	7.74	0.236
Palmitate.....	0.0045	7.77	7.76	0.058
Stearate.....	0.0018	7.77	7.77	0.023
Temperature 35°				
	gm.	gm.	gm.	gm.
Laurate.....	0.0630	7.76	7.70	0.805
Myristate.....	0.0287	7.75	7.72	0.373
Palmitate.....	0.0066	7.74	7.74	0.085
Stearate.....	0.0024	7.76	7.76	0.031
Temperature 50°				
	gm.	gm.	gm.	gm.
Laurate.....	0.0963	7.70	7.60	1.267
Myristate.....	0.0440	7.67	7.63	0.577
Palmitate.....	0.0116	7.67	7.66	0.151
Stearate.....	..	..	..	..



TABLE XV.  
*Solubility of Magnesium Salts in Methyl Alcohol.*

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15°				
	gm.	gm.	gm.	gm.
Laurate.....	0.0862	7.96	7.87	1.095
Myristate.....	0.0151	7.94	7.89	0.571
Palmitate.....	0.0180	7.93	7.92	0.227
Stearate.....	0.0066	7.93	7.92	0.084
Temperature 25°				
	gm.	gm.	gm.	gm.
Laurate.....	0.0863	7.88	7.79	1.108
Myristate.....	0.0594	7.84	7.78	0.763
Palmitate.....	0.0261	7.88	7.85	0.336
Stearate.....	0.0078	7.84	7.84	0.100
Temperature 51.5°				
	gm.	gm.	gm.	gm.
Palmitate....	0.0381	7.72	7.68	0.500
Stearate.....	0.0128	7.72	7.71	0.166

TABLE XVI.  
*Solubility of Magnesium Salts in Ether.*

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 25°				
	gm.	gm.	gm.	gm.
Laurate.....	0.0011	7.14	7.14	0.015
Myristate.....	0.0007	6.82	6.82	0.010
Palmitate.....	0.0003	7.49	7.49	0.004
Stearate.....	0.0002	7.47	7.47	0.003



TABLE XIX  
*Solubility of Magnesium Salts in Amyl Alcohol*

	Salt	Solution	Solvent	Salt in 100 gm solvent
Temperature 15°				
	gm	gm	gm	gm
Laurate	0.0151	7.91	7.90	0.191
Myristate	0.0065	7.93	7.92	0.086
Palmitate	0.0031	7.99	7.98	0.013
Stearate	0.0011	7.89	7.89	0.014
Temperature 25°				
Laurate	0.0186	7.89	7.88	0.236
Myristate	0.0115	7.92	7.91	0.145
Palmitate	0.0052	7.91	7.93	0.066
Stearate	0.0011	7.95	7.95	0.018
Temperature 35°				
Laurate	0.1152	7.89	7.78	1.481
Myristate	0.0344	7.90	7.86	0.438
Palmitate	0.0082	7.85	7.84	0.104
Stearate	0.0031	7.88	7.88	0.039
Temperature 50°				
Laurate	0.3647	7.85	7.49	4.869
Myristate	0.1446	7.79	7.64	1.893
Palmitate	0.0205	7.81	7.79	0.263
Stearate	0.0082	7.82	7.81	0.105

TABLE XX  
*Solubility of Magnesium Salts in Amyl Acetate*

	Salt	Solution	Solvent.	Salt in 100 gm solvent
Temperature 15°				
	gm	gm	gm.	gm
Laurate	0 0100	8 39	8 38	0 119
Myristate	0 0053	8 41	8 40	0 063
Palmitate	0 0033	8 43	8 42	0 039
Stearate	0 0025	8 42	8 42	0 029
Temperature 25°				
Laurate	0 0135	8 31	8 30	0 162
Myristate	0 0061	8 35	8 34	0 073
Palmitate	0 0038	8 37	8 36	0 045
Stearate	0 0027	8 37	8 37	0 030
Temperature 34.6°				
Laurate	0 0214	8 28	8 26	0 259
Myristate	0 0087	8 29	8 28	0 105
Palmitate	0 0047	8 30	8 30	0 057
Stearate	0 0038	8 29	8 29	0 046
Temperature 50°				
Laurate	0 1553	8 16	8 01	1 939
Myristate	0 0491	8 16	8 11	0 605
Palmitate	0 0176	8 14	8 12	0 216
Stearate	0 0094	8 17	8 16	0 115

The barium salts were made by adding an alcoholic solution of  $\text{Ba}(\text{OH})_2$  to the warm alcoholic solutions of the acids and then washing the precipitates formed with hot alcohol. Even upon exercising the greatest care, a small amount of  $\text{BaCO}_3$  was formed and precipitated from the alcoholic solution, but on account of the very slight solubility of the carbonate in the solvents used, no appreciable error in the solubility data is introduced from this source.

TABLE XXI.  
*Solubility of Barium Salts in Water.*

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15.3°				
	gm.	gm.	gm.	gm.
Laurate...	0 0008	9 89	9 89	0.008
Myristate . . . . .	0 0007	9 90	9.90	0 007
Palmitate . . . . .	0 0001	9 90	9.90	0 001
Stearate.....	0 0001	9 89	9.89	0 001
Temperature 50°				
Laurate.....	0 0011	9 77	9 77	0 011
Myristate.....	0 0009	8 72	8 72	0 010
Palmitate....	0 0007	9 81	9 81	0 007
Stearate.. . . .	0 0006	9 86	9 86	0.006

TABLE XXII  
*Solubility of Barium Salts in Ethyl Alcohol (Absolute).*

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 16 3°				
	gm	gm.	gm.	gm
Laurate.....	0 0008	7.82	7 82	0 010
Myristate.....	0 0007	7 81	7 81	0 009
Palmitate.....	0 0007	7.81	7 81	0.009
Stearate.....	0 0005	7 81	7 81	0 006
Temperature 25°				
Laurate.....	0 0008	7 78	7.78	0 010
Myristate.....	0 0009	7 79	7 79	0.011
Palmitate.....	0 0007	7 76	7 76	0 009
Stearate.....	0 0008	7 79	7 79	0 010
Temperature 35°				
Laurate.....	0 0010	7 72	7 72	0.013
Myristate.....	0 0010	7 73	7 73	0 013
Palmitate.....	0 0009	7 73	7 73	0.012
Stearate.....	0 0008	7 74	7 74	0 010
Temperature 50°				
Laurate.....	0.0005	7 60	7.60	0.007
Myristate.....	0 0003	7.67	7.67	0.004
Palmitate.....	0 0003	7.68	7.68	0.004
Stearate.....	0.0002	7.67	7.67	0.003

TABLE XXIII.

*Solubility of Barium Salts in Methyl Alcohol.*

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15°				
	gm.	gm.	gm.	gm.
Laurate . . . . .	0.0066	7.88	7.87	0.084
Myristate . . . . .	0.0045	7.91	7.91	0.057
Palmitate . . . . .	0.0036	7.91	7.90	0.045
Stearate . . . . .	0.0033	7.89	7.89	0.042
Temperature 25°				
Laurate . . . . .	0.0075	7.84	7.83	0.096
Myristate . . . . .	0.0055	7.84	7.83	0.070
Palmitate . . . . .	0.0040	7.86	7.86	0.051
Stearate . . . . .	0.0038	7.85	7.84	0.049
Temperature 35°				
Laurate . . . . .	0.0094	7.79	7.78	0.121
Myristate . . . . .	0.0068	7.79	7.78	0.087
Palmitate . . . . .	0.0058	7.80	7.80	0.074
Stearate . . . . .	0.0050	7.82	7.82	0.066
Temperature 50.5°				
Laurate . . . . .	0.0124	7.63	7.62	0.163
Myristate . . . . .	0.0083	7.71	7.70	0.108
Palmitate . . . . .	0.0068	7.71	7.70	0.088
Stearate . . . . .	0.0060	7.76	7.75	0.077

TABLE XXIV.

*Solubility of Barium Salts in Ether (Distilled over Sodium):*

	Salt	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 25°				
	gm.	gm.	gm.	gm.
Laurate.....	0.0005	6.97	6.97	0.007
Myristate....	0.0002	6.97	6.97	0.003
Palmitate ..	0.0001	7.00	7.00	0.001
Stearate.....	0.0001	6.92	6.92	0.001

TABLE XXV.  
*Solubility of Barium Salts in Amyl Alcohol.*

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 25°				
	gm	gm	gm.	gm.
Laurate.....	0 0007	7 93	7.93	0 009
Myristate ...	0 0007	7 72	7.72	0 009
Palmitate.....	0 0006	7 91	7.91	0.008
Stearate.....	0 0006	7.90	7.90	0 007

The lead salts were made by adding calculated amounts of lead acetate, dissolved in a mixture of alcohol and water to alcoholic solutions of the fatty acids. The stearate and palmitate precipitates were washed by decantation with boiling alcohol and finally on the filter. The laurate was washed with cold alcohol and the myristate recrystallized from boiling alcohol. The salts were all amorphous powders except the laurate which comes down crystalline.

TABLE XXVI.  
*Solubility of Lead Salts in Water.*

	Salt	Solution	Solvent.	Salt in 100 gm. solvent
Temperature 35°				
	gm.	gm.	gm	gm.
Laurate .	0 0009	9 85	9 85	0 009
Myristate	0 0005	9 85	9 85	0 005
Palmitate	0 0005	9 85	9 85	0 005
Stearate .	0 0005	9 85	9 85	0 005
Temperature 50°				
Laurate	0 0007	9 82	9 82	0 007
Myristate	0 0006	9 81	9 81	0 006
Palmitate .	0 0007	9 84	9 84	0 007
Stearate	0 0006	9 82	9 82	0 006

TABLE XXVII  
*Solubility of Lead Salts in Ethyl Alcohol (Absolute).*

	Salt	Solution.	Solvent	Salt in 100 gm solvent
Temperature 25°				
	gm.	gm.	gm.	gm.
Laurate	0 0007	7 80	7 80	0 009
Myristate	0 0003	7 80	7 80	0 004
Palmitate	0 0000	7 80	7 80	0 000
Stearate	0 0000	7 78	7 78	0 000
Temperature 35°				
Laurate	0 0025	7 74	7 74	0 032
Myristate	0 0003	7 73	7 73	0 004
Palmitate	0 0001	7 74	7 74	0 001
Stearate	0 0001	7 71	7 71	0 001
Temperature 50°				
Laurate	0 0202	7 67	7 64	0 264
Myristate	0 0040	7 66	7 66	0 052
Palmitate	0 0009	7 70	7 70	0 012
Stearate	0 0003	7 66	7 66	0 004



TABLE XXVIII.

*Solubility of Lead Salts in Methyl Alcohol.*

	salt	Solution	Solvent.	Salt in 100 gm. solvent.
Temperature 15°				
	gm.	gm.	gm.	gm.
Laurate.....	0 0018	7 91	7 90	0 061
Myristate.. . . .	0 0011	7 87	7 87	0 056
Palmitate..... . . .	0 0010	7 90	7 90	0 051
Stearate . . . . .	0 0031	7 90	7 90	0 039
Temperature 25°				
Laurate . . . . .	0 0075	7 85	7 81	0 096
Myristate..... . .	0 0061	7 85	7 85	0 078
Palmitate..... . . .	0 0051	7 87	7 86	0 069
Stearate . . . . .	0 0040	7 82	7 82	0 051
Temperature 35°				
Laurate . . . . .	0 0088	7 79	7 78	0 113
Myristate.....	0 0061	7 81	7 81	0 082
Palmitate... . . . .	0 0059	7 79	7 78	0 076
Stearate... . . . .	0 0018	7 81	7 81	0 062
Temperature 50°				
Laurate..... .	0 0216	7 73	7 71	0 280
Myristate.....	0 0092	7 70	7 69	0 119
Palmitate . . . . .	0 0072	7 72	7 71	0 093
Stearate. . . . .	0 0064	7 73	7 73	0 083

TABLE XXIX.

*Solubility of Lead Salts in Ether (Distilled over Sodium).*

	Salt	Solution	Solvent	Salt in 100 gm. solvent.
Temperature 14 5°				
	gm.	gm.	gm.	gm.
Laurate .	0 0008	7 04	7 04	0 010
Myristate.....	0 0009	6 96	6 96	0 013
Palmitate	0 0007	7 05	7 05	0 010
Stearate	0 0005	7 04	7 04	0 007

TABLE XXXIV.

*Solubility of Silver Salts in Methyl Alcohol*

	Salt	Solution	Solvent	Salt in 100 gm solvent
Temperature 15°				
	gm	gm	gm	gm
Laurate	0 0058	7 88	7 87	0 074
Myristate	0 0050	7 89	7 89	0 063
Palmitate	0 0048	7 91	7 90	0 060
Stearate	0 0040	7 91	7 91	0 051
Temperature 25°				
Laurate	0 0056	7 84	7 84	0 072
Myristate	0 0053	7 87	7 86	0 067
Palmitate	0 0046	7 88	7 88	0 059
Stearate	0 0041	7 87	7 87	0 052
Temperature 35°				
Laurate	0 0061	7 78	7 77	0 078
Myristate	0 0055	7 80	7 80	0 071
Palmitate	0 0048	7 79	7 79	0 062
Stearate	0 0043	7 80	7 79	0 055
Temperature 50°				
Laurate	0 0064	7 70	7 70	0 083
Myristate	0 0056	7 72	7 71	0 073
Palmitate	0 0051	7 71	7 71	0 066
Stearate	0 0046	7 71	7 71	0 060

TABLE XXXV

*Solubility of Silver Salts in Ether (Distilled over Sodium)*

	Salt	Solution	Solvent	Salt in 100 gm solvent
Temperature 15°				
	gm	gm	gm	gm
Laurate	0 0007	6 99	6 99	0 010
Myristate	0 0006	7 02	7 02	0 009
Palmitate	0 0006	6 75	6 75	0 009
Stearate	0 0005	7 04	7 01	0 007

## CONCLUSIONS.

From the above tables it is seen that the solubility of all the salts of the four fatty acids in the various solvents tried is only slight, but that considerable differences are found not only among the several salts in the same solvent, but also for the same salts in the different solvents.

The solubility in any case rarely exceeds 1 per cent, but was found to vary between 6 per cent and virtual insolubility. Methyl alcohol was found to be the best general solvent for this class of substances.

The lithium salts were found to be about three times as soluble in methyl alcohol and acetone as the magnesium salts, while the latter are more soluble in ethyl alcohol than the former.

It is also seen that the lithium salts are a great deal more soluble in water than the magnesium salts, but the difference is not a constant ratio for the different temperatures.

The beryllium salts of the fatty acids were made, but contrary to expectations we found that the basic salts, rather than the normal, were formed.



# THE SEPARATION OF LAURIC AND MYRISTIC ACIDS FROM EACH OTHER AND FROM MIXTURES OF OTHER FATTY ACIDS.\*

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With the preceding paper<sup>1</sup> as a basis, the authors have succeeded in working out what appear to be practical methods for the isolation of lauric and myristic acids when present in mixtures of the higher fatty acids.

By way of introduction it should be said that, in general, all fatty acids occurring in nature may be classified in three main groups: the saturated, unsaturated, and hydroxy acids.<sup>2</sup> The acids of these three major groups may be subdivided into subgroups, either with reference to properties or molecular structure. The saturated fatty acids are best adapted to a property classification, while the acids of the other two groups may be classified with respect to their molecular structure. According to this grouping the hydroxy acids are subdivided into mono-, di-, tri-, etc., hydroxy acids, and the unsaturated acids into those having a double bond, such as oleic acid; a triple bond, such as stearolic acid; and a double and triple bond, such as ricinostearolic acid. The property relations of these subgroups have not been worked out satisfactorily.

The differential grouping of the saturated acids, with which this paper has to deal, is based on property relations, which may be formulated as follows: *Group I*, all the lower members up to and including caprylic acid. These are all capable of distillation at

\* This investigation was carried out at the Nevada Agricultural Experiment Station with funds obtained under the Adams Act.

<sup>1</sup> Jacobson, C. A., and Holmes, A., *J. Biol. Chem.*, 1916, xxv, 29.

<sup>2</sup> This is on the assumption that the amino and other substituted fatty acids are excluded.

atmospheric pressure without decomposition, and are more or less easily soluble in water. The Reichert-Meissl number is a fairly accurate criterion for the quantity of Group I acids in a given sample. *Group 2*, all the acids between and including capric and stearic acids. These are more or less soluble in cold and hot 90 per cent alcohol, but a regular gradation in solubility is found between the upper and lower members of the group. They cannot be distilled at ordinary pressures without decomposition, although they are volatile with steam. *Group 3*, all acids above stearic acid. They are practically insoluble in cold 90 per cent alcohol, and in general not volatile with steam.

For the separation of the fatty acids into these groups, as well as for the isolation of individual members of each group, a large number of proposed methods are to be found in the literature. A discussion of all those not bearing more or less directly upon the separations in Group 2 of the saturated acids, will be omitted.

This grouping is arbitrary, and the authors have been unable to find any property that would exactly fit one group to the exclusion of those adjacent. To illustrate, if we were to distill a mixture of the saturated fatty acids of Groups 1 and 2, we should find that certain amounts of some of the so called soluble volatile fatty acids of Group 1 would remain behind, and certain fractions of a few of the acids of Group 2 would be found in the distillate.

The Reichert-Wollny process<sup>3</sup> is perhaps the best for determining the acids of Group 1, and the Gusserow-Varrentrapp, lead-salt-ether method<sup>4</sup> the best for separating the unsaturated acids from the acids of this group. Various other methods for these separations have been proposed, but certain undesirable features accompany all of them.

The quantitative separation of the acids of Group 2 from those of Group 3 of this series is also fraught with considerable difficulty, but the method of dissolving in boiling 90 per cent alcohol and cooling to room temperature, using the proper concentration of the solution, would appear to be the best at present.

For the separation of individual members of Group 2, to which lauric and myristic acids belong, various methods have been proposed, such as the fractional precipitation of their salts, the frac-

<sup>3</sup> Reichert, E., and Wollny, R., *Analyst*, 1900, xxv, 309.

<sup>4</sup> Varrentrapp, F., *Ann. Chem.*, 1840, xxxv, 197.

tional distillation *in vacuo* of the acids, and of their methyl and ethyl esters.

Gsell<sup>5</sup> worked out a method for separating capric acid from the other members of this group by making anhydrides of the acids in ethereal solution, dissolving in pyridine, and pouring this solution into water. He says the mixed anhydride of capric acid will remain dissolved, while the normal anhydrides of the other acids will be precipitated. Lewkowitsch<sup>6</sup> attempted to make anhydrides of these acids, but from his results he concluded that "the acid values found for the products of the interaction of acetic anhydride and fatty acids lose every quantitative meaning."

For the separation of stearic and palmitic acids, Kreis and Hafner<sup>7</sup> have worked out a modification of Hehner and Mitchell's method,<sup>8</sup> but it is not found to be entirely satisfactory.

The authors tested out experimentally two well known methods for the separation of the fatty acids in this group, but the results were far from satisfactory. The methods tried were Facchini and Dorta's,<sup>9</sup> and Partheil and Feri's.<sup>10</sup> The former method depends upon the difference in solubility of the potassium soaps in acetone at different temperatures, while the latter depends upon the difference in solubility of the potassium soaps in 50 per cent and 100 per cent alcohol.

Following are the results from the latter method: 0.500 gm. of each of lauric, myristic, palmitic, and stearic acids were dissolved in alcohol, neutralized with 0.2 N alcoholic potash, the volume was made up to 200 cc. and sufficient water added to bring the concentration of alcohol to 50 per cent by volume. The acids were precipitated by adding 10 per cent lithium acetate in 50 per cent alcoholic solution. The mixture was then heated until all the salts passed into solution, and set aside to cool. The crystallized salts thus obtained were filtered off, giving Precipitate I in Table I, and the filtrate was treated with 10 per cent aqueous lead acetate, filtered, washed, and the fatty acids were liberated with hydrochloric acid.

<sup>5</sup> Gsell, J., *Chem. Zeit.*, 1907, xxxi, 100.

<sup>6</sup> Lewkowitsch, J., *J. Chem. Soc.*, 1890, Proc. 92.

<sup>7</sup> Kreis, H., and Hafner, A., *Ber. chem. Ges.*, 1903, xxxvi, 2766.

<sup>8</sup> Hehner, O., and Mitchell, C. A., *Analyst*, 1896, xxi, 320.

<sup>9</sup> Facchini, S., and Dorta, W., *Chem. Rev. Fett.-u. Harz-Ind.*, 1912, xix, 77.

<sup>10</sup> Partheil, A., and Feri, F., *Arch. Pharm.*, 1903, cxxi, 545.

The acids thus obtained constitute Precipitate II. Precipitate I was boiled with absolute alcohol, the solution allowed to cool, the precipitate formed was filtered and washed, which constitutes Precipitate III. Precipitate IV was obtained by evaporating the filtrate from Precipitate III to dryness.

The fatty acids of Precipitates II and III were liberated from their lithium soaps by hydrochloric acid, washed, dried, and weighed. The neutralization values of the acids from these pre-

TABLE I.

	a.	b	c
Precipitate I.			
Yield { Gm. . . . .	1 300	0.888	1.092
Per cent . . . . .	63.4	43.3	53.3
	(With 100 cc. absolute alcohol.)	(With 150 cc. absolute alcohol.)	(With 150 cc. absolute alcohol.)
Precipitate II.			
Yield.....	0 603 gm.	0.996 gm.	0.765 gm.
Neutralization value . . . . .	266	252.5	258
Per cent laurie acid.....	60	19	35
“ “ myristic “ . . . . .	40	81	65
Precipitate III.			
Yield.....	0.940 gm.	0.716 gm.	0.913 gm.
Neutralization value... . .	214	207.9	209.1
Per cent stearic acid.....	29	52	46
“ “ palmitic “ . . . . .	71	48	54
Precipitate IV.			
Yield.....	0.339 gm.	0.156 gm.	0.149 gm.
Neutralization value.....	232	219	236
Per cent palmitic acid....	52	100	37
“ “ myristic “ . . . . .	48	—	63

cipitates were obtained and will also be found in Table I. It was learned that the amount of lithium acetate added influenced the character of the soap mixtures in the different precipitates. Under Column a will be found the results obtained when a large excess of lithium acetate was added; under Column b, when 10 cc. of a 10 per cent solution were added; and under Column c, when 20 cc. of the acetate were used.

The results here obtained indicate that no quantitative separa-



tion can be obtained by this method, but that a small amount of pure palmitic acid was isolated in Precipitate IV by using 10 cc of lithium acetate solution and 150 cc. of absolute alcohol as the solvent. The yield, however, was only 31.2 per cent in this case. The results from the method of Fatchin and Dorta were no more encouraging.

From an inspection of the solubility data of the salts of lauric, myristic, palmitic, and stearic acids given in the preceding paper,<sup>1</sup> we learn that only two possible methods for separations appear. They are as follows. First, it is shown that lithium laurate is about five times as soluble in water as lithium myristate, the next higher salt, at the four temperatures investigated, and this suggests the possibility of extracting the laurate from a mixture of lithium salts with water or the precipitation of the three salts from water, retaining the laurate in solution. Second, it is seen that the solubility of magnesium myristate is about five times as great as magnesium palmitate in absolute alcohol at 15° and 25°, suggesting the possibility of separating the myristate from the two higher salts after the lauric acid has been removed as the lithium salt by the foregoing method.

To test these possibilities, the following experiments were carried out.

*Experiment 1.*—0.5 gm of each of the four fatty acids were weighed out, mixed, and dissolved in strong alcohol, and the solution was neutralized with 0.2 N alcoholic potassium hydroxide. The mixed acids had a neutralization value of 231.3, calculated value for the mixture 235.8. The alcohol was evaporated off and the soaps were taken up in 300 cc water, after which a calculated amount of lithium acetate was added to make the lithium salts. The mixture was boiled, cooled, and filtered. The precipitate thus obtained weighed 1.65 gm, or was a yield of 80 per cent. The filtrate was evaporated to three-fourths of its volume, cooled, filtered, and the fatty acid liberated with dilute hydrochloric acid. The acid, weighing 0.155 gm, had a neutralization value of 271, whereas the corresponding value for lauric acid is 280.5.

These results suggested the possibility of a separation by this method upon varying the conditions somewhat.

*Experiment 2.*—Again, a mixture of 0.5 gm of each of the four acids was converted into potassium soaps and, after evaporating off the alcohol, was taken up in 400 cc of water and precipitated with a slight excess of lithium acetate solution. In this case the precipitated lithium salts

weighed 1.562 gm., equivalent to 1.527 gm. of acid mixture, which was 76.3 per cent of the original acid mixture, whereas 75 per cent should have been obtained if the separation had been perfect.

The filtrate was concentrated to one-half its volume, cooled, and filtered, yielding a precipitate weighing 0.208 gm., and the acid obtained from this precipitate yielded a neutralization value of 278.3, and had a melting point of 43.2°, whereas the acid obtained by evaporating the filtrate to dryness gave a neutralization value of 271.2 and the same melting point. Calculated for lauric acid: 280.5 and 43.6° respectively.

The water-insoluble salts from this separation yielded an acid mixture having a neutralization value of 217.5, whereas a mixture of equal quantities of myristic, palmitic, and stearic acids gives a neutralization value of 220.9.

It was next tried to separate myristic acid from a mixture of the three higher acids of this group by means of the difference in the solubility of their magnesium salts in absolute alcohol, but the experiments resulted unsuccessfully, doubtless because of the influence of one salt exerted upon the solubility of the others, in this solvent. Instead of absolute alcohol, 50 per cent alcohol was used as the medium in which the precipitation of the magnesium salts was carried out, according to the conditions given in the following experiment.

*Experiment 3.*—0.5 gm. each of magnesium myristate, palmitate, and stearate were digested with 60 cc. of 50 per cent alcohol for 2 hours at 60°, then cooled, and filtered. The fatty acids were liberated from the filtrate, washed, dried, and weighed, giving 0.270 gm. with a melting point of 48.6° and a neutralization value of 243. Since the yield was only about one-half of what it should be, the insoluble salts were again extracted with alcohol in the same manner, yielding an insoluble residue weighing 0.028 gm. This time the acids were liberated from the insoluble residue and after being recrystallized from 60 per cent alcohol showed a melting point of 56.5° and a neutralization value of 209.3. Calculated for a mixture of equal parts of stearic and palmitic acids: 56.4° and 208.3 respectively.

The acids obtained from this insoluble residue must therefore be a mixture of equal quantities of stearic and palmitic acids, showing that the myristic acid had been almost completely removed.

A subsequent experiment using 100 cc. of 50 per cent alcohol did not yield as good results as the foregoing but a fairly good separation was effected.

During the course of the investigation it was noticed that after

neutralizing an alcoholic solution of these fatty acids with alcoholic potassium hydroxide, and allowing the solution to stand over night, a white crystalline and a gelatinous precipitate appeared in the flask the next morning. These two were so different in properties that they could be separated by decantation, but upon investigation it was found that both forms yielded practically the same fatty acid mixture. Whether they represented allotropic modifications or simply crystalline and colloidal forms of the potassium salts of the fatty acid mixture was left undetermined.

#### CONCLUSION.

In the present paper the authors have presented a method for the separation of lauric acid when present in a mixture of myristic, palmitic, and stearic acids, and a method for the separation of myristic acid from a mixture in which it occurs, together with palmitic and stearic acids. These methods are based upon the differences of the solubility of their lithium and magnesium salts in water and 50 per cent alcohol.



# CHANGES IN THE $H^+$ AND $OH^-$ CONCENTRATION WHICH TAKE PLACE IN THE FORMATION OF CERTAIN PROTEIN COMPOUNDS.

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(Received for publication, February 26, 1916.)

It has been known for some time that certain proteins combine with other proteins to form compound proteins. Kossel<sup>1</sup> states that the protamines in weakly alkaline solutions combine with proteins to form compounds which precipitate under proper conditions. Kutscher,<sup>2</sup> working with albumoses obtained from Witte's peptone prepared compounds not only with protamine but also with various other proteins. While studying the properties of the histones, Bang<sup>3</sup> was able to confirm the observations of Kutscher and further showed that various members of this group combine with proteins to form compounds. Kossel and Kutscher<sup>4</sup> surmised that histones (on account of the high content of basic substances) might be a combination of protamine with other proteins but later Kossel and Pringle<sup>5</sup> showed that this view was incorrect since in the first step in the peptic digestion of histones, histopeptone is formed, whereas protamine-protein combinations are split into protamine and protein. Hunter<sup>6</sup> prepared a number of compounds of protamine with various proteins, such combinations as elupein-casein, elupein-gelatin, and elupein-destin being obtained. He further determined the proportion of the two substances which unite to form the compound protein. Gay and Robertson,<sup>7</sup> studying the antigenic properties of

<sup>1</sup> Kossel, A., *Deutsch. med. Woch.*, 1894, xx, 147.

<sup>2</sup> Kutscher, F., *Z. physiol. Chem.*, 1897, xxiii, 115.

<sup>3</sup> Bang, I., *Z. physiol. Chem.*, 1899, xxvii, 463.

<sup>4</sup> Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, 1900-01, xxi, 165.

<sup>5</sup> Kossel, A., and Pringle, H., *Z. physiol. Chem.*, 1906, xlix, 301.

<sup>6</sup> Hunter, A., *Z. physiol. Chem.*, 1907, liii, 526.

<sup>7</sup> Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1912, xvi, 479.

The method used to determine the  $H^+$  concentrations was essentially the same as used by Robertson<sup>20,21</sup> and Robertson and Schmidt,<sup>22</sup> and described by Schmidt and Finger<sup>15</sup> and Robertson.<sup>24</sup> Hydrogen was generated by the electrolysis of a 6 per cent by volume solution of concentrated  $H_2SO_4$  and passed over heated platinized asbestos to rid it of any oxygen or oxygen compounds. The hydrogen then passed through the solution at two points, through a nozzle at the bottom of the cell and

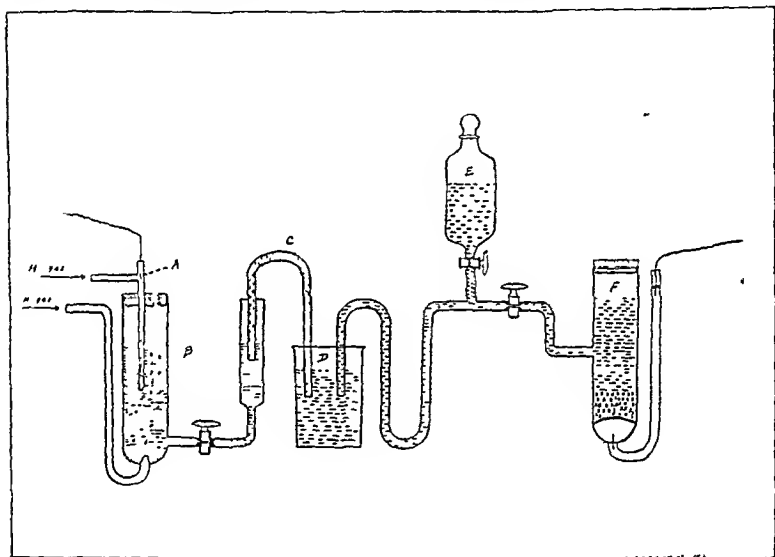


FIG. 1. A, platinum gauze electrode; B, titration cell; C, agar tube saturated with KCl; D, beaker with 0.1 N KCl; E, separatory funnel with 0.1 N KCl; F, 0.1 N KCl-HgCl-Hg-electrode.

through a Cottrell gauze electrode.<sup>15, 25</sup> In this way the three phases, platinum, hydrogen, and solution were brought into intimate contact. A calomel electrode,<sup>26</sup> using 0.1 N KCl, was used as the other extremity of the chain. The apparatus is shown in Fig. 1. A is the gauze electrode dipping into the solu-

<sup>21</sup> Robertson, *Die physikalische Chemie der Proteine*, Dresden, 1912, 417.

<sup>22</sup> Robertson, *J. phys. Chem.*, 1907, xi, 442. Loomis, N. E., Dissertation, Johns Hopkins Univ., 1911, 22. Loomis, N. E., and Aree, S. F., *Am. Chem. J.*, 1911, xlv, 602.

<sup>26</sup> Richards, T. W., *Z. physik. Chem.*, 1897, xxiv, 39.

tion contained in cell B, D a beaker containing 0.1 N KCl, and F the calomel electrode. Connection with cell B is made by means of an agar tube saturated with KCl which eliminates contact potential.<sup>27, 28</sup> To eliminate contamination of the solution in cell B, the stop-cock connecting the side arm with the cell was kept closed except during the measurement of the potential and likewise the agar tube was dipped into the solution in the side arm only while readings were being taken. Through a hole in the stopper of cell B any amount of a solution "b" could be introduced. Measurement of the potential was made on a 100 cm. bridge using a sensitive Leeds and Northrup galvanometer<sup>29</sup> as zero instrument. Three Edison-Lalande cells connected in series furnished current through the potentiometer wire and the E.M.F. was checked against a Weston cell after each reading. The protein solutions "a" and "b" as well as the ganze electrode were separately saturated with hydrogen before bringing them together, the necessity for this having been shown by Robertson<sup>20</sup> and by Desha and Acree.<sup>31</sup> Hydrogen was allowed to bubble through the solution from 45 to 60 minutes before determining the E.M.F., and this was likewise done after each addition of solution "b". All determinations were made at room temperature. To prevent foaming a few drops of octyl alcohol were floated on the surface of the solution in B after the introduction of the electrode. The  $H^+$  and  $OH^-$  concentrations corresponding to the E.M.F.'s were taken from tables previously calculated by Schmidt.<sup>31</sup>

Since it is not feasible to determine  $H^+$  or  $OH^-$  concentrations in ammoniacal solutions by means of the gas chain, the experi-

<sup>27</sup> Bjerrum, N, *Z physik Chem*, 1905, lxx, 128. Loomis, N E, Dissertation, Johns Hopkins Univ, 1911, 31. Loomis, N E, and Acree, S. F., *Am. Chem J.*, 1911, xlv, 585. Clark, F. W., Myers, C N, and Acree, S. F., *J. phys. Chem*, 1916, xx, 241. Bjerrum, *Z Electrochem*, 1911, xvii, 389. See also Cumming, A. C, and Abegg, R, *Z Electrochem*, 1907, xiii, 17.

<sup>28</sup> This may not be strictly true, but since we are not concerned with absolute values but merely with changes in voltage this factor may be neglected.

<sup>29</sup> Kindly loaned by the Physics Department

<sup>30</sup> Desha, L. J, and Acree, S. F., *Am. Chem. J*, 1911, xlv, 638

<sup>31</sup> Schmidt, C. L. A., *Univ. California Publications, Physiology* 1905-10, iii, 101.

ments were made with compounds of globin. As stated by Bang<sup>3</sup> and Robertson,<sup>8</sup> precipitation of the compound protein takes place in slight excess of NaOH or KOH. Globin was prepared according to a modification of the method described by Robertson.<sup>32</sup> Two preparations were made, one precipitated by  $NH_4Cl$  from an ammoniacal solution and the other by alcohol and ether from HCl solution. These correspond to Preparations I and II described by Robertson. Preparation II is soluble in water without addition of acid or alkali. The casein employed was Eimer and Amend's ("*nach Hammarsten*") which had been further purified according to Robertson.<sup>33</sup> Deuteroalbumose was made from Witte's peptone as described by Kutscher.<sup>2</sup> The nucleic acid was a preparation extracted by Dr. A. E. Taylor from the sperm of the Pacific Coast salmon (probably according to the method of Altmann).<sup>10</sup> The bile salts were prepared from ox bile according to the method of Plattner.<sup>34</sup>

The following is a tabular representation of the results obtained in the experiment. In the first column are given the volumes of the titrating solution "*b*" which were added to a given amount of solution "*a*". The second column shows the E.M.F. determined after the establishment of equilibrium. The  $H^+$  and  $OH^-$  concentrations corresponding to the E.M.F.'s are given in the next two columns. In the last column are given the calculated possible errors in determining the  $H^+$  concentration on the assumption that the determination of the E.M.F.'s was accurate to a millivolt.

Curves showing either the change in the  $H^+$  or the  $OH^-$  concentration which took place during the titration as determined by experiment are plotted and given below. These will be referred to as titration curves. The first experiments were carried out to determine what effect was produced on the  $H^+$  concentration by simple dilution and by salting out with those salts which might be formed as intermediate products of the reaction; so that, if possible, a distinction between salting out and true protein com-

<sup>32</sup> Robertson, *J. Biol. Chem.*, 1912-13, xiii, 455.

<sup>33</sup> Robertson, *J. Biol. Chem.*, 1906-07, ii, 317; *J. phys. Chem.*, 1910, xiv, 534.

<sup>34</sup> Plattner, E. A., *J. prakt. Chem.*, 1847, xl, 129.



pound formation might be made. The results are given in Tables I, II, and III and shown graphically in Fig. 2. The change in  $H^+$  concentration is a direct function of the titrating solution and the curves are straight lines, despite the fact that with both  $NH_4Cl$  and  $KCl$  a precipitate is obtained. In these instances we apparently have either a purely physical phenomenon or a chemical one which involves no sudden change in the  $H^+$  concentration such as to give a break in the curve. The precipitation of proteins by inorganic salts has been studied extensively

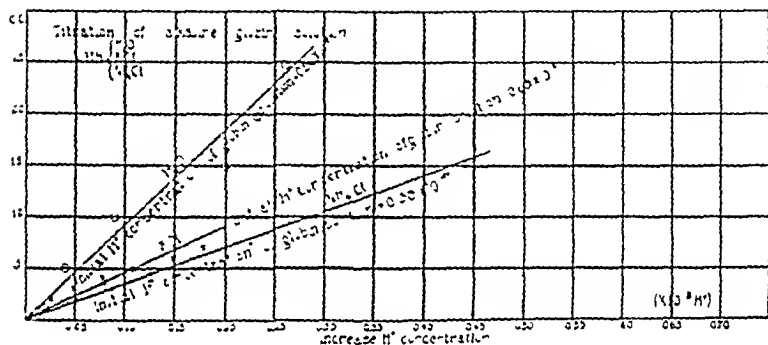


FIG. 2.

TABLE I.

*Titration of Alkaline Globin (Preparation I) Solution with Water*

Concentration of globin, 0.5 per cent. 0.25 gm. globin dissolved by 8 cc 0.1 N KOH, then titrated back with 7 cc. 0.1 N HCl. 50 cc. globin solution titrated. No precipitate produced

H <sub>2</sub> O added.	E.M.F.	H <sup>+</sup>	OH <sup>-</sup>	Possible error in H <sup>+</sup>
cc.	volts			
0	0.861	$0.80 \times 10^{-3}$	$0.80 \times 10^{-3}$	$\pm 0.06 \times 10^{-3}$
1	0.856	$0.98 \times 10^{-3}$	$0.65 \times 10^{-3}$	$0.08 \times 10^{-3}$
5	0.850	$0.12 \times 10^{-3}$	$5.3 \times 10^{-5}$	$0.01 \times 10^{-3}$
10	0.842	$0.17 \times 10^{-3}$	$3.8 \times 10^{-5}$	$0.01 \times 10^{-3}$
15	0.830	$0.28 \times 10^{-3}$	$2.3 \times 10^{-5}$	$0.03 \times 10^{-3}$
20	0.827	$0.31 \times 10^{-3}$	$2.1 \times 10^{-5}$	$0.02 \times 10^{-3}$
25	0.825	$0.34 \times 10^{-3}$	$1.9 \times 10^{-5}$	$0.03 \times 10^{-3}$

TABLE II.

*Titration of Alkaline Globin (Preparation I) Solution with a Saturated Solution of KCl.*

Concentration of globin, 0.5 per cent. 0.25 gm globin dissolved by 8 cc. 0.1 N KOH, then titrated back with 7 cc. 0.1 N HCl. 50 cc globin solution titrated. Precipitate produced

KCl solution added	E M F.	$H^+$	$OH^-$	Possible error in $H^+$
cc	volts			
0	0.867	$0.63 \times 10^{-3}$	$1.0 \times 10^{-3}$	$\pm 0.05 \times 10^{-3}$
1	0.859	$0.87 \times 10^{-3}$	$0.74 \times 10^{-3}$	$0.07 \times 10^{-3}$
2	0.859	$0.87 \times 10^{-3}$	$0.74 \times 10^{-3}$	$0.07 \times 10^{-3}$
3	0.853	$0.11 \times 10^{-3}$	$5.8 \times 10^{-6}$	$0.01 \times 10^{-3}$
4	0.847	$0.14 \times 10^{-3}$	$4.6 \times 10^{-6}$	$0.01 \times 10^{-3}$
5	0.842	$0.17 \times 10^{-3}$	$3.8 \times 10^{-6}$	$0.01 \times 10^{-3}$
6	0.837	$0.21 \times 10^{-3}$	$3.1 \times 10^{-6}$	$0.02 \times 10^{-3}$
7	0.834	$0.24 \times 10^{-3}$	$2.7 \times 10^{-6}$	$0.02 \times 10^{-3}$

TABLE III.

*Titration of Alkaline Globin (Preparation I) Solution with a 0.25 Per Cent Solution of  $NH_4Cl$ .*

Concentration of globin, 0.5 per cent. 0.25 gm globin dissolved by 8 cc. 0.1 N KOH, then titrated back with 7 cc. 0.1 N HCl. 50 cc. globin solution titrated. Precipitate produced

$NH_4Cl$ solution added	E M F	$H^+$	$OH^-$	Possible error in $H^+$
cc	volts			
0	0.927	$0.58 \times 10^{-10}$	$1.1 \times 10^{-4}$	$\pm 0.05 \times 10^{-10}$
0.5	0.911	$0.11 \times 10^{-3}$	$5.8 \times 10^{-3}$	$0.01 \times 10^{-3}$
1.0	0.893	$0.22 \times 10^{-3}$	$2.9 \times 10^{-3}$	$0.01 \times 10^{-3}$
1.5	0.874	$0.48 \times 10^{-3}$	$1.3 \times 10^{-3}$	$0.04 \times 10^{-3}$
2.0	0.869	$0.58 \times 10^{-3}$	$1.1 \times 10^{-3}$	$0.04 \times 10^{-3}$
2.5	0.868	$0.61 \times 10^{-3}$	$1.1 \times 10^{-3}$	$0.05 \times 10^{-3}$
3.0	0.857	$0.94 \times 10^{-3}$	$0.68 \times 10^{-3}$	$0.07 \times 10^{-3}$
3.5	0.859	$0.87 \times 10^{-3}$	$0.74 \times 10^{-3}$	$0.07 \times 10^{-3}$
4.5	0.848	$0.13 \times 10^{-3}$	$4.9 \times 10^{-6}$	$0.01 \times 10^{-3}$
5.5	0.843	$0.17 \times 10^{-3}$	$3.9 \times 10^{-6}$	$0.02 \times 10^{-3}$
7.5	0.835	$0.23 \times 10^{-3}$	$2.8 \times 10^{-6}$	$0.02 \times 10^{-3}$
9.5	0.831	$0.27 \times 10^{-3}$	$2.4 \times 10^{-6}$	$0.03 \times 10^{-3}$
14.5	0.819	$0.43 \times 10^{-3}$	$1.5 \times 10^{-6}$	$0.03 \times 10^{-3}$

by Hardy,<sup>35</sup> Pauli,<sup>36</sup> Hofmeister,<sup>37</sup> Galeotti,<sup>38</sup> and many others, and is discussed at length by Robertson.<sup>39</sup>

The titration of globin in acid and alkali solutions by alkali and acid respectively was next studied. For this purpose Preparation I, dissolved by a small amount of alkali, and Preparation II, which is water-soluble and acid in reaction, were used. During

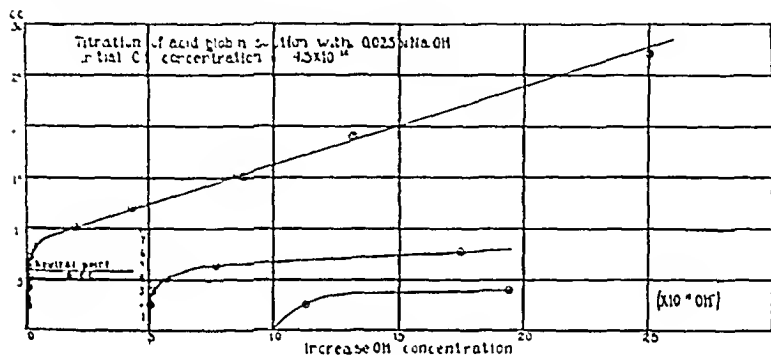


FIG. 3.

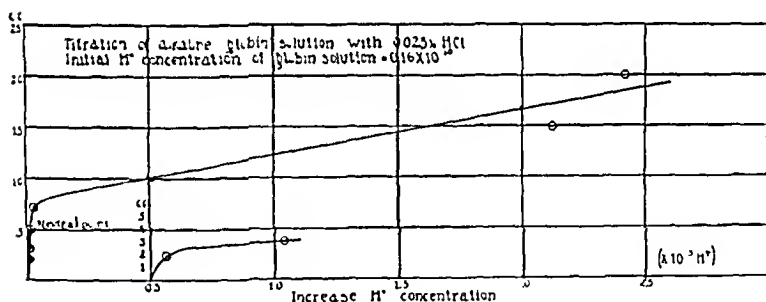


FIG. 4

the titration the protein was precipitated and redissolved by excess of acid or alkali respectively. The results are given in

<sup>35</sup> Hardy, W. B., *J. Physiol.*, 1905-06, xxviii, 251

<sup>36</sup> Pauli, W., *Beitr. chem. Phys u Path.*, 1906, vii, 531

<sup>37</sup> Hofmeister, F., *Arch. exp. Path.*, 1888, xxiv, 247; 1889, xxv, 1; 1890, xxvii, 395; 1891, xxviii, 210.

<sup>38</sup> Galeotti, G., *Z. physiol. Chem.*, 1903-04, xl, 492, 1904, xli, 330.

<sup>39</sup> Robertson, *Die physikalische Chemie der Proteine*, Dresden, 1912, 84.

TABLE VII.

*Titration of Alkaline Globin (Preparation I) Solution with a Solution of Casein (Neutral to Litmus).*

Concentration of globin, 0.5 per cent. 0.25 gm. globin dissolved by 3 cc. 0.1 N KOH, then titrated back with 7.1 cc. 0.1 N HCl. 50 cc. globin solution titrated. Concentration of casein in titrating solution, 0.5 per cent. 0.25 gm. casein dissolved by 3 cc. 0.1 N KOH, then titrated back with 1.5 cc. 0.1 N HCl. Slight precipitate produced.

Casein solution added	E. M. F.	$H^+$	$OH^-$	Possible error in $H^+$
cc.	volts			
0	0.843	$0.17 \times 10^{-8}$	$3.9 \times 10^{-6}$	$\pm 0.02 \times 10^{-8}$
1	0.833	$0.24 \times 10^{-8}$	$2.6 \times 10^{-6}$	$0.01 \times 10^{-8}$
2	0.831	$0.26 \times 10^{-8}$	$2.4 \times 10^{-6}$	$0.02 \times 10^{-8}$
3	0.821	$0.40 \times 10^{-8}$	$1.6 \times 10^{-6}$	$0.03 \times 10^{-8}$
4	0.820	$0.41 \times 10^{-8}$	$1.6 \times 10^{-6}$	$0.03 \times 10^{-8}$
5	0.818	$0.45 \times 10^{-8}$	$1.4 \times 10^{-6}$	$0.04 \times 10^{-8}$
10	0.804	$0.78 \times 10^{-8}$	$0.82 \times 10^{-6}$	$0.06 \times 10^{-8}$
15	0.803	$0.81 \times 10^{-8}$	$0.79 \times 10^{-6}$	$0.06 \times 10^{-8}$
25	0.801	$0.88 \times 10^{-8}$	$0.73 \times 10^{-6}$	$0.07 \times 10^{-8}$

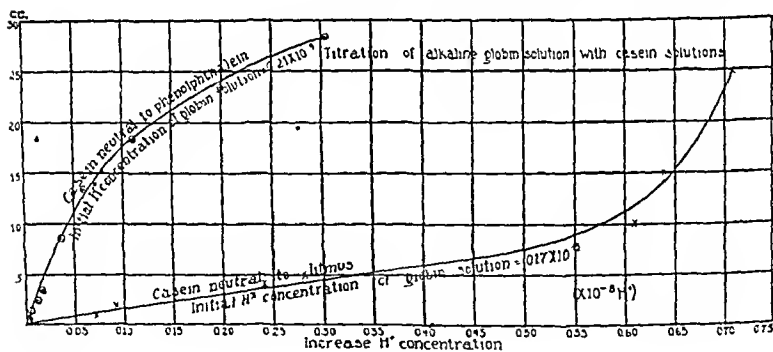


FIG. 5.

As previously stated, both Bang and Robertson obtained compounds of globin and casein which they state were precipitated in alkaline solution. On attempting to duplicate this work, no precipitate was obtained though a compound between globin and casein is formed as shown by the data in Tables VI and VII and represented graphically in Fig. 5. The compound formed is apparently soluble. However, on titrating an acid solution of

globin with a solution of casein (neutral to phenolphthalein) a precipitate was obtained and the titrating curve (see data in Table VIII and Fig. 6) shows that a compound was formed.

TABLE VIII.

*Titration of Acid Globin (Preparation II) Solution with a Solution of Casein.*

Concentration of globin, 0.2 per cent. Globin dissolved in water and 1 cc. 0.1 N NaOH added. Concentration of casein (neutral to phenolphthalein), 0.5 per cent. 50 cc. globin solution titrated. Precipitate produced.

Casein solution added.	E.M.F.	H <sup>+</sup>	OH <sup>-</sup>	Possible error in H <sup>+</sup>
cc.	volts			
0	0.552	$0.18 \times 10^{-3}$	$3.5 \times 10^{-11}$	$\pm 0.01 \times 10^{-3}$
1	0.577	$0.67 \times 10^{-4}$	$0.96 \times 10^{-10}$	$0.03 \times 10^{-4}$
2	0.609	$0.19 \times 10^{-4}$	$3.4 \times 10^{-10}$	$0.01 \times 10^{-4}$
4	0.611	$0.15 \times 10^{-4}$	$1.2 \times 10^{-10}$	$0.01 \times 10^{-4}$
6	0.623	$0.11 \times 10^{-4}$	$6.0 \times 10^{-10}$	$0.01 \times 10^{-4}$
8	0.637	$0.61 \times 10^{-5}$	$1.1 \times 10^{-9}$	$0.03 \times 10^{-5}$
11	0.650	$0.36 \times 10^{-5}$	$1.8 \times 10^{-9}$	$0.02 \times 10^{-5}$
16	0.685	$0.90 \times 10^{-6}$	$0.71 \times 10^{-8}$	$0.04 \times 10^{-6}$
21	0.707	$0.37 \times 10^{-6}$	$1.7 \times 10^{-8}$	$0.03 \times 10^{-6}$

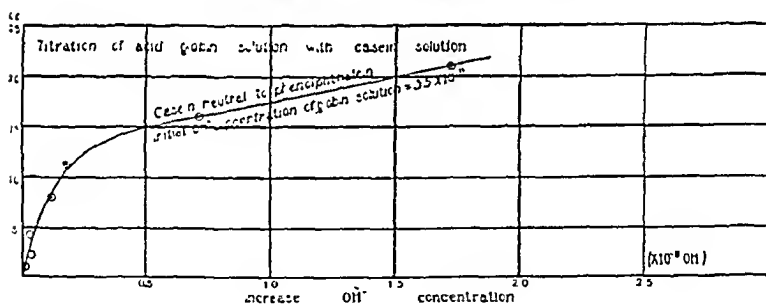


FIG. 6.

Apparently Robertson had used Globin Preparation II, which, being precipitated from an acid solution by alcohol and ether, was the acid compound of globin; and by dissolving in weak alkali, Robertson had merely partly neutralized the acid combined with the globin (not enough to cause precipitation of the globin) so that his solution was still acid, instead of alkaline as one is led to believe from his description. This was likewise true of the work

of Bang, who prepared globin according to the method of Schulz.<sup>42</sup> Determination of the initial acidity of the globin solution would have served as a criterion for the duplication of the work.

TABLE IX.

*Titration of Acid Globin (Preparation II) Solution with a Solution of Sodium Nucleate.*

Concentration of globin, 0.2 per cent. Globin dissolved in water without addition of acid or alkali. Concentration of nucleic acid, 0.4 per cent. Nucleic acid neutralized by NaOH. 50 cc. globin solution titrated. Pre-precipitate produced.

Sodium nucleate solution added.	E M F.	$H^+$	$OH^-$	Possible error in $H^+$
cc	volts			
0	0.488	$0.23 \times 10^{-2}$	$2.8 \times 10^{-12}$	$\pm 0.01 \times 10^{-2}$
2	0.486	$0.25 \times 10^{-2}$	$2.5 \times 10^{-12}$	$0.01 \times 10^{-2}$
4	0.504	$0.12 \times 10^{-2}$	$5.2 \times 10^{-12}$	$0.01 \times 10^{-2}$
8	0.525	$0.53 \times 10^{-3}$	$1.2 \times 10^{-11}$	$0.02 \times 10^{-3}$
12	0.536	$0.34 \times 10^{-3}$	$1.9 \times 10^{-11}$	$0.02 \times 10^{-3}$
16	0.560	$0.13 \times 10^{-3}$	$4.9 \times 10^{-11}$	$0.01 \times 10^{-3}$
20	0.572	$0.82 \times 10^{-4}$	$0.78 \times 10^{-10}$	$0.03 \times 10^{-4}$
25	0.585	$0.49 \times 10^{-4}$	$1.3 \times 10^{-10}$	$0.03 \times 10^{-4}$

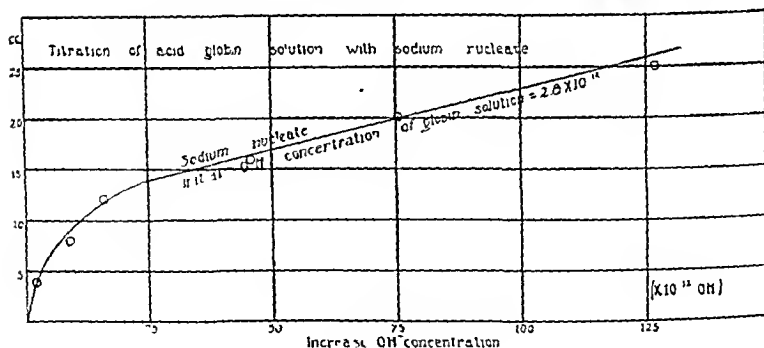


FIG. 7.

Globin also forms compounds with nucleic acid and taurocholic acid, precipitation taking place in an acid solution. The titration curve of globin with nucleic acid (see Table IX and Fig. 7) is very similar to that obtained on titration of acid glo-

<sup>42</sup> Schulz, R. N. Z. physiol. Chem., 1898, xxiv, 449.

bin with casein. The titration curve of acid globin with bile salts (see Tables X and XI, and Fig. 8) shows a sharp break at the

TABLE X.

*Titration of Acid Globin (Preparation II) Solution with a Solution of Bile Salts.*

Concentration of globin, 0.2 per cent. Globin dissolved in water without addition of acid or alkali. Concentration of bile salts in aqueous solution, 0.4 per cent. Precipitate produced

Bile salts solution added.	L.M.F.	H <sup>+</sup>	OH <sup>-</sup>	Possible error in H <sup>+</sup>
cc.	volts			
0	0.181	$0.27 \times 10^{-2}$	$2.3 \times 10^{-12}$	$\pm 0.01 \times 10^{-2}$
2	0.498	$0.16 \times 10^{-2}$	$1.1 \times 10^{-12}$	$0.01 \times 10^{-2}$
4	0.503	$0.13 \times 10^{-2}$	$5.0 \times 10^{-12}$	$0.01 \times 10^{-2}$
5	0.507	$0.11 \times 10^{-2}$	$5.9 \times 10^{-12}$	$0.01 \times 10^{-2}$
7	0.511	$0.93 \times 10^{-3}$	$0.69 \times 10^{-11}$	$0.04 \times 10^{-3}$
10	0.508	$0.11 \times 10^{-2}$	$6.1 \times 10^{-12}$	$0.01 \times 10^{-2}$
13	0.512	$0.89 \times 10^{-3}$	$0.72 \times 10^{-11}$	$0.04 \times 10^{-3}$
16	0.517	$0.73 \times 10^{-3}$	$0.88 \times 10^{-11}$	$0.03 \times 10^{-3}$
21	0.510	$0.29 \times 10^{-3}$	$2.2 \times 10^{-11}$	$0.01 \times 10^{-3}$

TABLE XI.

*Titration of Acid Globin (Preparation II) Solution with a Solution of Bile Salts.*

Concentration of globin, 0.2 per cent. Globin dissolved in water without addition of acid or alkali. Concentration of bile salts in aqueous solution, 0.4 per cent. Several drops 0.1 N NaOH added to the solution of bile salts to make it slightly alkaline. Precipitate produced during titration.

Bile salts solution added.	L.M.F.	H <sup>+</sup>	OH <sup>-</sup>	Possible error in H <sup>+</sup>
cc.	volts			
0	0.515	$0.79 \times 10^{-3}$	$0.81 \times 10^{-11}$	$\pm 0.04 \times 10^{-3}$
2	0.521	$0.63 \times 10^{-3}$	$1.0 \times 10^{-11}$	$0.03 \times 10^{-3}$
5	0.525	$0.53 \times 10^{-3}$	$1.2 \times 10^{-11}$	$0.02 \times 10^{-3}$
8	0.524	$0.55 \times 10^{-3}$	$1.2 \times 10^{-11}$	$0.02 \times 10^{-3}$
10	0.526	$0.51 \times 10^{-3}$	$1.3 \times 10^{-11}$	$0.02 \times 10^{-3}$
13	0.532	$0.40 \times 10^{-3}$	$1.6 \times 10^{-11}$	$0.02 \times 10^{-3}$
17	0.536	$0.34 \times 10^{-3}$	$1.9 \times 10^{-11}$	$0.02 \times 10^{-3}$
21	0.542	$0.27 \times 10^{-3}$	$2.4 \times 10^{-11}$	$0.01 \times 10^{-3}$
25	0.548	$0.21 \times 10^{-3}$	$3.0 \times 10^{-11}$	$0.01 \times 10^{-3}$

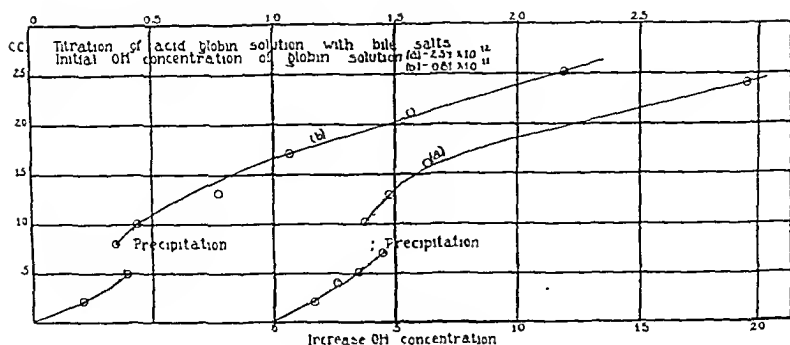


FIG. 8.

point where the compound protein starts to precipitate, and the slope of the curve changes. This is to be expected since we are titrating with a mixture of two salts, sodium taurocholate and sodium glycocholate. Globin also forms a compound with deutoalbumose, the latter acting as a weak acid. The titration curve (Fig. 9 and Table XII) indicates the formation of a compound, the proportion being roughly two parts of globin to one of albumose.

TABLE XII.

*Titration of Alkaline Globin (Preparation I) Solution with a Solution of Deutoalbumose.*

Concentration of globin, 0.5 per cent. 0.25 gm. globin dissolved by 8 cc. 0.1 N KOH, then titrated back with 7.1 cc. 0.1 N HCl. Concentration of deutoalbumose, 0.6 per cent. 50 cc. globin solution titrated. Precipitate produced.

Albumose solution added.	E.M.F.	$H^+$	$OH^-$	Possible error in $H^+$
cc.	volts			
0	0.835	$0.23 \times 10^{-8}$	$2.8 \times 10^{-6}$	$\pm 0.02 \times 10^{-8}$
1	0.813	$0.54 \times 10^{-8}$	$1.2 \times 10^{-6}$	$0.04 \times 10^{-8}$
2	0.792	$0.13 \times 10^{-7}$	$5.1 \times 10^{-7}$	$0.01 \times 10^{-7}$
4	0.793	$0.12 \times 10^{-7}$	$5.3 \times 10^{-7}$	$0.01 \times 10^{-7}$
7	0.792	$0.13 \times 10^{-7}$	$5.1 \times 10^{-7}$	$0.01 \times 10^{-7}$
15	0.786	$0.16 \times 10^{-7}$	$4.0 \times 10^{-7}$	$0.01 \times 10^{-7}$
20	0.779	$0.21 \times 10^{-7}$	$3.0 \times 10^{-7}$	$0.01 \times 10^{-7}$



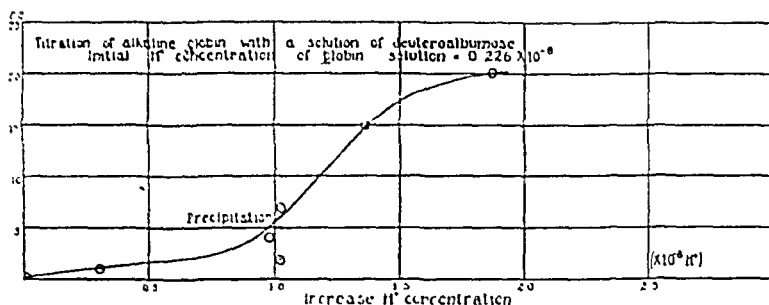


FIG. 9.

## SUMMARY.

1. Use of the gas chain has been made to follow the changes in the  $H^+$  and  $OH^-$  concentration in titrating acid and alkaline solutions of globin with solutions of other proteins and of precipitating inorganic salts.

2. Titration curves showing the changes in  $H^+$  and  $OH^-$  concentration during titration have been plotted which indicate that true protein compounds of globin are formed, some of which may precipitate at a proper acidity or alkalinity, while others may be soluble.

3. A distinction can be made by means of the gas chain between the salting out of globin from solution by inorganic salts and the precipitation of a compound protein.

4. Determination of the  $H^+$  or  $OH^-$  concentration in protein solutions used in the preparation of compound proteins serves as a criterion for the duplication of such work.

I am indebted to Professors F. P. Gay and T. Brailsford Robertson for the interest taken in this work, and to the George Williams Hooper Foundation for Medical Research for financial aid in carrying out the investigation.



# THE FATE OF NORMAL $\alpha$ -AMINOCAPROIC ACID IN THE PHLORHIZINIZED DOG.

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As a result of the work of Lusk,<sup>1</sup> Ringer,<sup>1</sup> and Dakin,<sup>2</sup> we know that a number of the amino-acids obtained from proteins can give rise to an increased excretion of glucose when administered to phlorhizinized dogs. One of the amino-acids that has not yet been investigated is normal  $\alpha$ -aminocaproic acid, or norleucine, first isolated from brain tissue by Abderhalden and Weil.<sup>3</sup>

It is the purpose of this paper to present the results of an attempt to ascertain the effect of the administration of this substance upon the excretion of nitrogen and glucose in the phlorhizinized dog. Two experiments were made with each of the dextrorotatory and racemic acids and one with the levorotatory acid.

## EXPERIMENTAL.

Phlorhizin was administered in daily doses of 1 gm. each in 7 or 8 cc. of cottonseed oil. In Experiment 5 the dog was prepared with epinephrin, as recommended by Sansum and Woodyatt.<sup>4</sup> The urine was collected by catheter and the bladder washed with sterile 2 per cent boric acid solution at 12 hour intervals. Glucose was determined according to Benedict,<sup>5</sup> by polarization before and after fermentation and by fermentation (Lohnstein), gross urea nitrogen by the urease method,<sup>6</sup> and

<sup>1</sup> Literature reviewed by Lusk, G., *Ergebn. Physiol.*, 1912, xii, 315.

<sup>2</sup> Dakin, H. D., *J. Biol. Chem.*, 1913, xiv, 321.

<sup>3</sup> Abderhalden, E., and Weil, A., *Z. physiol. Chem.*, 1913, lxxxiv, 39.

<sup>4</sup> Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 1.

<sup>5</sup> Benedict, S. R., *J. Biol. Chem.*, 1911, ix, 57.

<sup>6</sup> It should be noted that it was necessary to allow a much longer time (3 hours) for the action of the urease in these urines than is recommended by Plimmer and Skelton (*Biochem. J.*, 1914, viii, 70).

aminonitrogen by the method of Benedict and Murlin.<sup>7</sup> In all but Experiment 5 the acetone was determined by titration with iodine after distillation and the  $\beta$ -hydroxybutyric acid by Shaffer's method applied to the ethereal extract. In Experiment 5 Shaffer's method<sup>8</sup> was used directly.

*Preparation of the Normal  $\alpha$ -Aminocaproic Acid.*—The amino-acid was prepared by the method described by Abderhalden, Froehlich, and Fuchs.<sup>9</sup> There were obtained 290 gm. of bromocaproic acid from 192 gm. of caproic acid but only 60 gm. of recrystallized normal  $\alpha$ -aminocaproic acid. This is a much smaller yield than that reported by Abderhalden, Froehlich, and Fuchs. The melting point given by them is 297–300°. This preparation melted at 294–296° and contained 10.55 per cent nitrogen (calculated, 10.66 per cent). The formyl derivative was then prepared and the optical isomers separated by means of their brucine salts. The free amino-acids were isolated in the usual manner.

*Normal d- $\alpha$ -Aminocaproic Acid.*—0.5427 gm. in 12.5 cc. HCl (s.g. 1.11), in a 2 dm. tube, rotated the plane of polarization +1.48°,  $[\alpha]_D + 17.05^\circ$ . The substance was, therefore, made up of 84.1 per cent pure d-acid and 15.9 per cent l-acid.<sup>10</sup>

*Normal l- $\alpha$ -Aminocaproic Acid.*—0.4823 gm. in 12.5 cc. HCl (s.g. 1.11), in a 2 dm. tube, rotated the plane of polarization -1.74°,  $[\alpha]_D - 22.6^\circ$ . This preparation was, therefore, 91.6 per cent pure l-acid and 8.4 per cent d-acid.<sup>10</sup>

The amino-acid was administered subcutaneously in three or four doses at intervals of 1 or 2 hours. Attempts were made to isolate unchanged amino-acid from the urine by means of its insoluble copper salt. In only one experiment was this successful. In calculating the amount of glucose derived from the administered substance, it was assumed, as it has been by previous workers, that all of the nitrogen of the amino-acid appeared in the urine in the experimental period, or in that immediately following.

<sup>7</sup> Benedict, S. R., and Murlin, J. R., *J. Biol. Chem.*, 1913-14, xvi, 356.

<sup>8</sup> Shaffer, P. A., and Marriott, W. McK., *J. Biol. Chem.*, 1913-14, xvi, 276.

<sup>9</sup> Abderhalden, E., Froehlich, C., and Fuchs, D., *Z. physiol. Chem.*, 1913, lxxxvi, 454.

<sup>10</sup> Schulze, E., and Likiernik, A., *Z. physiol. Chem.*, 1893, xvii, 523.

## DISCUSSION.

The results of the experiments indicate that both optical isomers are attacked in the organism of the dog, the dextro form being only slightly more resistant. The amount of "extra glucose" excreted was, in four experiments, approximately equal to that of the amino-acid administered. In the two instances in which the amount of extra glucose was smaller than this, there was unquestionably a considerable excretion of unchanged amino-acid. In Experiment 4, in which 3.45 gm. of extra glucose were obtained from 5.76 gm. of the *d*-acid, the increase in the amino nitrogen of the urine indicated that 15 per cent of the amino-acid appeared in the urine unchanged. In the one experiment in which the dog was prepared with epinephrin, only 3.6 gm. of extra glucose were obtained after the administration of 14.7 gm. of *dl*-acid. Unfortunately the amount of amino-acid available does not permit of a repetition of the experiment. However, it is scarcely possible that the extra glucose obtained in the other experiments should have been derived from the glycogen remaining in the body after 3 or 4 days' treatment with phlorhizin. Normal  $\alpha$ -aminocaproic acid has no narcotic or other toxic action. It seems quite as likely that treatment with epinephrin interferes with the formation of glucose from some amino-acids. The low ratios reported by Sansum and Woodyatt<sup>4</sup> indicate that this may be the case. Certainly a considerable quantity of unchanged amino-acid was eliminated in the urine in this experiment. 35 cc. of the urine of the experimental period (total volume 800 cc.) were precipitated with  $\text{CuSO}_4$ , keeping the reaction neutral. The precipitate was filtered out, washed, and decomposed with  $\text{H}_2\text{S}$ . The liquid was filtered from the  $\text{CuS}$ , evaporated, and treated with five volumes of alcohol. Characteristic crystals of the amino-acid were obtained. These were filtered out, washed, dissolved in water, and the copper salt was again precipitated. Its appearance was quite characteristic. It weighed 88 mg. Unfortunately the material was lost in the process of analysis but there can be little doubt that it was the copper salt of normal  $\alpha$ -aminocaproic acid. The amount recovered corresponds, in the urine of the entire period, to 1.3 gm. of amino-acid, or 9 per cent of the amount injected. It is almost certain that a larger amount was excreted

estimation that are employed or not as the case requires. But in any event the phosphoric acid is finally precipitated as magnesium ammonium phosphate, which is decomposed by heating and weighed as magnesium pyrophosphate. From the weight of the pyrophosphate the corresponding weight of phosphoric acid is calculated.

The uniform rejection by analysts of such a substance as crystalline magnesium ammonium phosphate in favor of magnesium pyrophosphate, in spite of the fact that the one substance precedes the other in the analytical scheme and must of necessity transmit to the other any errors committed in its manipulation, is a matter that renders one suspicious and suggests caution. Nevertheless, the following data demonstrate very clearly that if there is any choice between the two substances, aside from economy of time, it is in favor of the crystalline compound.

To prove the admissibility of magnesium ammonium phosphate it is necessary, and sufficient, to establish three points: (a) that magnesium ammonium phosphate can be brought to a constant weight; (b) that the substance can be easily and completely removed from a filter paper when dry; (c) that the substance possesses exactly the composition expressed by the formula  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  or at least that it will produce the exact amount of magnesium pyrophosphate that this formula demands. These three points are taken up in order as follows.

a. In order to meet the conditions of a phosphorus partition study the specimens of magnesium ammonium phosphate employed in this work were prepared from the hydrolytic products of yeast nucleic acid. Five portions of the substance were heated with 5 per cent sulfuric acid from 1 to 5 hours and after removal of the guanine with ammonia, the phosphoric acid was precipitated with magnesia mixture, using such precaution as to insure the formation of crystalline magnesium ammonium phosphate. The five precipitates were filtered on ashless papers, washed carefully, allowed to dry in the room for 19 hours, and weighed. In order to see if any further change in weight would occur at the room temperature, the filters were allowed to stand several days longer and weighed from time to time. The results are given in Table I.

TABLE I.

	Weights of filter papers containing magnesium ammonium phosphate dried at room temperature.				
	I.	II.	III.	IV.	V.
	gm.	gm.	gm.	gm.	gm.
After 19 hrs.....	0 7297	0 7535	0 7882	0.7695	0.7254
" 5 " longer. . .	0 7284	0 7527	0.7875	0.7690	4.6669
" 21 " " . .	0 7271	0 7514	0 7864	0 7679	4.6668
" 8 " " .	0 7268	0 7510	0 7860	0 7677	4 6668
" 17 " " . . . .	0 7272	0 7212	0 7863	0.7680	4.6668

It will be seen that up to the last period a slow but continual loss in weight has occurred uniformly in all the specimens. But this weight is lost from the filter paper, not from the precipitate, for at the beginning of the second period, most of Precipitate V was transferred to a platinum crucible lid and weighed. The constancy of this weight throughout shows that in the other four specimens it was not the magnesium ammonium phosphate whose weight altered.

This change in the weight of a filter paper, which may amount to 2 mg. in 24 hours, can scarcely affect the results obtained by weighing an empty filter paper a few minutes after the phosphate precipitate has been dusted off. It seems to be caused by a change of atmospheric conditions (temperature and moisture).

The following series shows that the filters may increase in weight at the room temperature. No. VI is a total phosphorus determination after all organic matter had been destroyed.

TABLE II.

	I	II.	III.	IV	V	VI
	gm	gm	gm	gm	gm	gm
After 16 hrs. in air...	0 9516	0 8327	0 8618	0 8185	0 7400	1 0386
After 24 hrs. longer in air.....	0 9528	0 8337	0 8631	0 8201	0 7419	1 0395

The next series shows that the filters may increase in weight even when they are allowed to remain in the thermostat at 40° for 48 hours. No. VI is a determination of total phosphorus and No. V which remained nearly constant, was weighed on a platinum crucible lid.

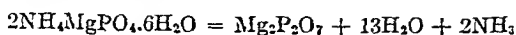
TABLE III.

	I.	II.	III.	IV.	V.	VI.
	gm	gm.	gm.	gm.	gm.	gm.
After 16 hrs. in air	0.8241	0.8342	0.8000	0.7903	5.1175	1.0619
2 days longer at 40°	0.8250	0.8352	0.8011	0.7912	5.1171	1.0630

The figures show that while a washed filter paper may vary slightly, ammonium magnesium phosphate maintains a constant weight when allowed to remain in the air or even at a temperature of 40°. The results given are extreme cases. As a rule the filter papers do not vary either way as much as 1 mg. in 24 hours.

b. In order to find whether small particles adhere to the filter when crystalline magnesium ammonium phosphate is removed, ten of the filters were dusted free from the salt and ashed in a weighed porcelain crucible. The total ash weighed 17 mg. or about 1.7 mg. for each filter paper. The ash was dissolved by long digestion with hot nitric acid and from the diluted solution the phosphoric acid was precipitated by the addition of ammonium nitrate and treatment with ammonium molybdate. The yellow ammonium phosphomolybdate was converted into magnesium ammonium phosphate which weighed about 5 mg. or about 0.5 mg. for each filter paper. Thus, if one uses an ashless filter paper and determines the phosphoric acid as magnesium pyrophosphate the final weighing is liable to be 1.7 mg. too high even though the paper has been well washed. Less than one-third of this error will be caused by the adhesion of magnesium ammonium phosphate to a filter paper.

c. As far as the composition of magnesium ammonium phosphate is of concern in this connection, it is only necessary to show that the substance produces the amount of magnesium pyrophosphate required by the equation:



Specimens of magnesium ammonium phosphate obtained in such experiments as those described above were heated to a constant weight in a weighed porcelain crucible. The determinations were made successively in the same crucible, and the negligible differ-



ences found are probably due to the difficulty of weighing the porcelain crucible.

TABLE IV.

	I.	II.	III.	IV.	V.
Magnesium ammonium phosphate used, gm....	0.3772	0.5619	0.4848	0.4467	0.5741
Magnesium pyrophosphate obtained, gm....	0.1703	0.2542	0.2642	0.2013	0.2594
Percentage found.....	45.15	45.21	45.18	45.06	45.20
Percentage required....	45.09	45.09	45.09	45.09	45.09

The results given in this paper seem to show that in the quantitative determination of phosphoric acid, the conversion of magnesium ammonium phosphate into magnesium pyrophosphate is a superfluous operation.

After this paper had been sent to the publishers, a letter was received from Dr. Louis Baumann (State University of Iowa), which contained the following data.

The magnesium contained in a solution prepared by dissolving pure magnesium ribbon in dilute hydrochloric acid was precipitated as the triple phosphate, then filtered on a weighed ash-free filter paper. The precipitate and paper were dried for 48 hours in the laboratory atmosphere. Results in triplicate:



Calculated for 0.0155 magnesium.	Found.
0.1564	0.1566
	0.1565
	0.1561



# HYDROLYSIS OF YEAST NUCLEIC ACID WITH AMMONIA.

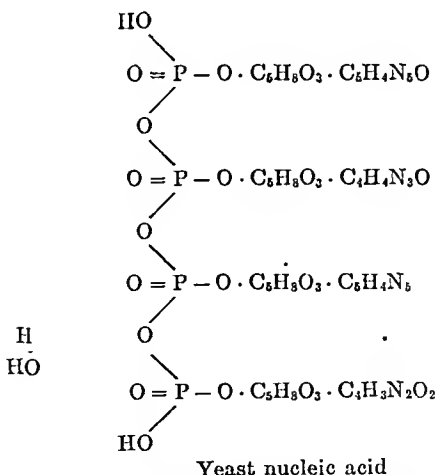
BY WALTER JONES AND HILDEGARDE C. GERMANN.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University, Baltimore.)

(Received for publication, April 1, 1916.)

About two years ago, Thannhauser<sup>1</sup> studied the action of duodenal juice on yeast nucleic acid and found that a tri-nucleotide is formed which has the composition represented by the formula  $C_{32}H_{43}P_3O_{23}N_{15}$ , is levorotatory to polarized light, and forms a crystalline brucine salt of the composition  $C_{32}H_{43}P_3O_{23}N_{15}(C_{23}H_{26}N_2O_4)_6$  which melts at 200–205°. From the tri-nucleotide he prepared the three nucleosides guanosine, adenosine, and cytidine.

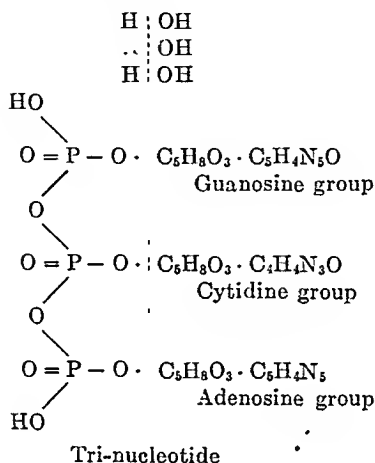
The article is written with a little caution, evidently because the analytical values of the substance do not lead to the formula that we would assign to a tri-nucleotide derived from yeast nucleic acid. Thannhauser therefore names his substance *Triphosphonucleinsäure*. But if he holds the prevailing well grounded opinion of the constitution of yeast nucleic acid he must believe that his substance is formed from yeast nucleic acid according to the following scheme,



<sup>1</sup> Thannhauser, S. J., *Z. physiol. Chem.*, 1914, xci, 329.

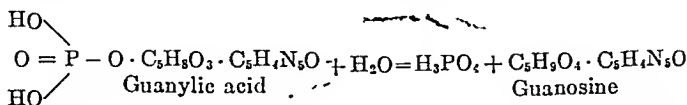
in which yeast nucleic acid is represented as decomposing by hydrolysis into a tri-nucleotide which contains the guanine, cytosine, and adenine groups, and a mono-nucleotide which contains the uracil group.

The formation of the three nucleosides from the tri-nucleotide must be represented as follows.



The following criticism of Thannhauser's results will make much clearer the matters that are to be discussed in this paper.

(a) The preparation of three nucleosides from the tri-nucleotide is of no consequence if evidence can be produced to show that this supposed tri-nucleotide is guanylic acid, a mono-nucleotide which has been prepared from yeast nucleic acid by the action of ferments,<sup>2</sup> and which can yield only one nucleoside,



Thannhauser states that he obtained three nucleosides, but if his results are carefully examined it will be seen that he obtained neither adenosine nor cytidine, but two picrates, one of which could be picric acid as far as his nitrogen determination is concerned.

<sup>2</sup> Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71.

(b) A tri-nucleotide containing groups of guanine ( $C_5H_5N_3O$ ), cytosine ( $C_4H_5N_3O$ ), and adenine ( $C_5H_5N_3$ ) must contain thirteen atoms of nitrogen and therefore cannot be correctly represented by the formula  $C_{32}H_{19}P_3O_{23}N_{13}$  which contains fifteen atoms of nitrogen.

(c) The analytical data given for the crystalline brucine salt of the tri-nucleotide answer as well for the crystalline brucine salt<sup>3</sup> of guanylic acid.

	Required for $C_{32}H_{19}N_3PO_4$ (C <sub>32</sub> H <sub>19</sub> N <sub>3</sub> O) <sub>4</sub> : (brucine salt of guanylic acid):	Required for $C_{32}H_{19}N_{13}P_3O_{23}$ (C <sub>32</sub> H <sub>19</sub> N <sub>3</sub> O) <sub>4</sub> : (brucine salt of tri-nucleotide):	Found:
N.....	10.91	10.90	10.36
P.....	2.69	2.88	2.71
C.....	58.39	58.82	59.12
H.....	5.74	5.77	5.88

(d) The tri-nucleotide is levorotatory and forms a crystalline brucine salt which melts at 200–205°.

Guanylic acid also is levorotatory and forms a crystalline brucine salt that melts at 200–205°.

In a more recent communication, Thannhauser and Dorf-müller<sup>4</sup> state that the same tri-nucleotide can be prepared from yeast nucleic acid by chemical means. They heated yeast nucleic acid with ammonia in the autoclave at 120–125° and from the product they prepared a crystalline brucine salt that melts at 205° and has the composition represented by the formula,  $C_{32}H_{19}N_{13}P_3O_{23}(C_{22}H_{26}N_2O)_6$ . This brucine salt is stated to be identical with the brucine salt of the tri-nucleotide that Thannhauser had previously prepared from yeast nucleic acid by the action of duodenal juice.

A second brucine salt was also obtained. Its melting point and elementary analysis are given but no formula is assigned. No examination is made of the hydrolytic products of either substance, the principal purpose of the article being to show that the same tri-nucleotide is formed from yeast nucleic acid

<sup>3</sup> Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 421.

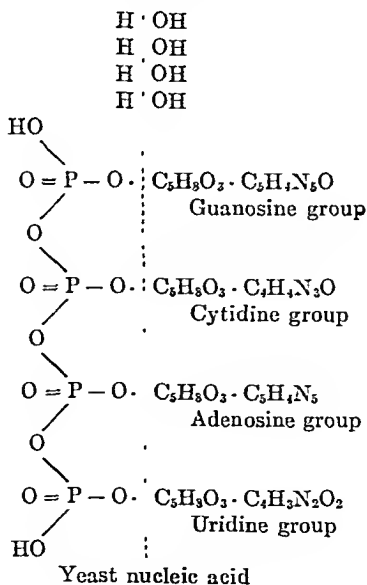
<sup>4</sup> Thannhauser, S. J., and Dorf-müller, G., *Z. physiol. Chem.*, 1915, xcv, 259. This number was issued Nov. 6, 1915, but has reached us only very recently.

by hydrolysis, whether the hydrolysis be brought about by ammonia in the autoclave at 120–125° or by the action of the ferments of duodenal juice.

In the last article, Thannhauser and Dorfmueller have described a method, long known in this laboratory, by which anyone can easily and quickly gain possession of the nucleotides of yeast nucleic acid. Moreover, a description has recently been given of an easily executed method by which the numerical indices of nucleotides may be found and their constitution determined.<sup>5</sup> We therefore wish to make a condensed statement of some of our results.

*Hydrolysis of Yeast Nucleic Acid with Ammonia at High Temperatures.*

Several years ago a serious attempt was made to prepare nucleosides from yeast nucleic acid by heating the substance in the autoclave with ammonia according to the method discovered and described by Levene and Jacobs.<sup>6</sup>



<sup>5</sup> Jones, W., *J. Biol. Chem.*, 1916, xxiv, p. iii.

<sup>6</sup> Levene and Jacobs, *Ber. chem. Ges.*, 1910, xliii, 3154.

The attempt to prepare nucleosides was made to gain possession of adenosine and to use the substance in studies of the action of ferments on combined adenine.<sup>7</sup>

Accordingly, yeast nucleic acid was heated in the autoclave with ammonia for 3½ hours at 175–180°, and as Levene and Jacobs specially enjoin, the temperature and time of heating were rigidly observed. A black tar was formed from which, of course, adenosine could not be prepared. After a number of discouraging attempts to prepare nucleosides in this way, some pure guanosine<sup>8</sup> was tested to see how the substance would behave when heated in the autoclave for 3½ hours at 175–180°. The substance was completely decomposed, and it became apparent that one could not expect to obtain nucleosides by heating nucleic acid at 175–180°. The experiment with nucleic acid was therefore made at lower temperatures and the time of heating was shortened to 2 hours.

At 165° the products were badly injured.

At 160° the products were considerably injured but nucleosides could be shown present.

At 150° the results were very satisfactory and nucleosides could be obtained from the product without difficulty.

At 145° all phosphoric acid is set free and can be precipitated directly with magnesia mixture. Under these conditions the production of nucleosides must be complete and there can be no reason for heating at any higher temperature.

Below 140° the liberation of phosphoric acid is partial and is less the lower the temperature.

At 120° no phosphoric acid is liberated so that nucleotides must be exclusively formed.

At 110° no phosphoric acid is liberated and still the nucleic acid is completely decomposed into soluble products.

We have chosen 115° as the temperature favorable to the formation of nucleotides. At this temperature there is no danger of forming nucleosides.

<sup>7</sup> Jones, *J. Biol. Chem.*, 1911, ix, 169. Amberg, S., and Jones, W., *Z. physiol. Chem.*, 1911, lxiii, 407.

<sup>8</sup> Guanosine had been plentifully obtained at this time by the action of ferments on yeast nucleic acid.

*Hydrolysis of Yeast Nucleic Acid with Ammonia at 115°.*

A solution of 100 gm. of yeast nucleic acid in 530 cc. of 2.5 per cent ammonia was heated in an autoclave at 115° for 1½ hours. The cooled product, which was strongly alkaline and could be shown to contain no nucleic acid, was treated with 95 per cent alcohol as long as a precipitate was formed and until the clear supernatant fluid showed a tendency to become cloudy. To bring about this condition about one and three-fourths volumes of alcohol are required.

This procedure was suggested by the difference in conduct of the two di-nucleotides of yeast nucleic acid when aqueous solutions of their potassium salts are poured into a large excess of alcohol. The potassium salt of one di-nucleotide is promptly precipitated by alcohol while the potassium salt of the other di-nucleotide remains suspended as an emulsion, and by this difference in conduct of their potassium salts, the two di-nucleotides can be separated from one another.<sup>9</sup>

The autoclave product that we are describing consists to a considerable extent, but not entirely, of the ammonium salts of the two di-nucleotides of yeast nucleic acid, and by treatment with alcohol, in the manner described, it may be separated rather sharply into two well characterized fractions, one of which is thrown down as a heavy gray precipitate while the other remains dissolved in the alcoholic fluid, if too much alcohol has not been added. The point at which the addition of alcohol should be discontinued is quite sharply indicated by a cloudy appearance of the liquid above the precipitate. This is caused by a little of the adenine di-nucleotide which is precipitated in colloidal form, and in such a case the cloud should be redissolved by the addition of a little water.

The precipitate is referred to as the guanine-cytosine fraction and contains only substances that produce guanine by acid hydrolysis. The alcoholic fluid is referred to as the adenine-uracil fraction and contains only substances that produce adenine by acid hydrolysis.

The alcoholic solution was filtered off and the precipitate was thoroughly washed with alcohol of the same strength as the filtrate. The alcohol washings were discarded.

<sup>9</sup> Jones and Richards, *J. Biol. Chem.*, 1915, xx, 25.



*The Guanine-Cytosine Fraction.*<sup>10</sup>

The heavy gray precipitate, formed by the addition of alcohol to the alkaline autoclave product, was dissolved in hot water and a small amount of dark, insoluble gelatinous material was removed with the centrifuge. By applying to the clear solution the procedure with lead acetate that is described in the next section a substance was finally obtained from which could be prepared a crystalline brucine salt melting at 200-205°. This crystalline compound is the brucine salt of guanylic acid. It produces the required amount of guanine, but not a trace of adenine, by hydrolysis with sulfuric acid. Its entire phosphoric acid is set free by heating with 5 per cent sulfuric acid, which proves that the substance does not contain a pyrimidine group.

The substance is undoubtedly the brucine salt of guanylic acid. Its melting point and method of preparation show with equal clearness that it is the brucine salt of Thannhauser and Dorf-müller's *Triphosphonucleinsäure*.

*The Adenine-Uracil Fraction.*

The perfectly clear alcoholic solution was diluted with an equal volume of boiling water, acidified with acetic acid, and treated with 25 per cent lead acetate as long as a precipitate was formed. After the hot material had cooled, the lead compound was filtered off, suspended in hot water, and decomposed with hydrogen sulfide. It is necessary to avoid a great excess of hydrogen sulfide and to boil the product finally until the vapor does not blacken lead acetate paper. Otherwise sulfur compounds are sure to be obtained. It is in fact advisable to use a slightly insufficient amount of hydrogen sulfide gas and remove the small amount of lead from the boiling hot filtered solution by the careful addition of hydrogen sulfide water.

The final filtrate from lead sulfide was evaporated to a syrup at 50° under diminished pressure and treated with several volumes of 95 per cent alcohol. A resin was precipitated which was ground with absolute alcohol to a heavy white powder and dried in a

<sup>10</sup> This fraction was examined by B. E. Read. He will publish later a full account of his work.

sulfuric acid desiccator. The substance is a di-nucleotide which contains groups of adenine and uracil.

Preparations of the substance often give a trace of guanine by hydrolysis with sulfuric acid. This is because a perfect separation of the ammonium salts with alcohol as described above was not effected. In such a case the di-nucleotide is dissolved in water and after making strongly alkaline with ammonia, the solution is treated with alcohol until the clear fluid shows a tendency to become cloudy. The small precipitate, which is a part of the guanosine-cytosine fraction, is filtered off, and after acidifying with acetic acid, the solution is diluted with hot water and treated with lead acetate. From the lead compound the di-nucleotide is prepared as already outlined.

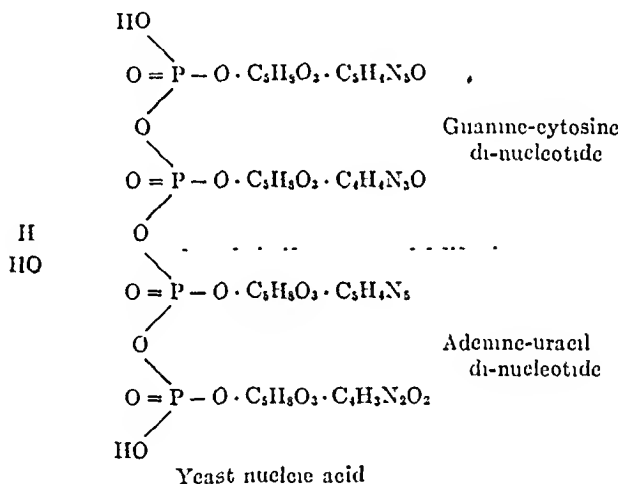
#### *The Adenine-Uracil Di-Nucleotide.*

The white granular powder prepared as described is adenine-uracil di-nucleotide and was obtained almost quantitatively from yeast nucleic acid. It contains the required amount of nitrogen and yields the required amount of adenine when hydrolyzed with dilute sulfuric acid. But it does not yield a trace of guanine. The partition of its phosphorus proves that the substance is a di-nucleotide. Exactly one-half of its phosphoric acid is easily split off and half is firmly bound; therefore as the nucleotide contains only one purine group, it can contain only one pyrimidine group.

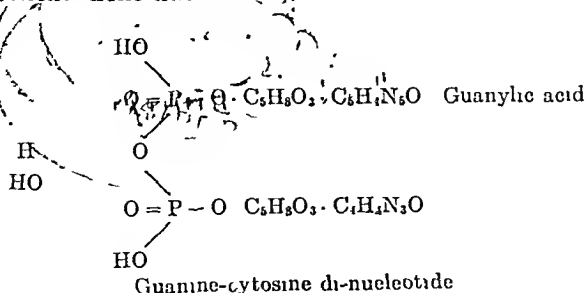
Some interesting results were obtained with a substance that was prepared in this laboratory several years ago by the action of a digested aqueous extract of pancreas on yeast nucleic acid. A provisional announcement had been made<sup>9</sup> that this preparation contained adenine-uracil di-nucleotide. By hydrolysis of the substance with sulfuric acid we obtained about five times as much adenine as guanine. A study of its phosphorus partition<sup>5</sup> gave exact di-nucleotide values. Evidently both the adenine and the guanine are in di-nucleotide combination.

## CONCLUSION.

There is no evidence to show that a tri-nucleotide can be produced from yeast nucleic acid either by the action of ferments or by hydrolysis with ammonia. All of the available evidence goes to show that when yeast nucleic acid undergoes hydrolysis, whether by the action of ammonia or under the influence of ferments, the substance is first decomposed into two di-nucleotides, one of which contains the adenine and uracil groups while the other contains the guanine and cytosine groups,



The adenine-uracil di-nucleotide is comparatively stable but the guanine-cytosine di-nucleotide easily undergoes hydrolysis into its component mono-nucleotides,



This last decomposition occurs to a considerable extent when the substance, *i.e.*, yeast nucleic acid, is heated for an hour or two with 2.5 per cent ammonia at 115° but to a much greater extent when the heating is done at 125°. At 125° the products begin to lose phosphoric acid and nucleosides are formed. At 145° all phosphoric acid is set free and the formation of nucleosides is complete.

## NOTE ON HYDROLYSIS OF YEAST NUCLEIC ACID IN THE AUTOCLAVE.

By P. A. LEVENE AND W. A. JACOBS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 18, 1916.)

It is customary in this laboratory to heat autoclaves by means of an oil bath, measuring the temperature of the oil bath. In a previous article<sup>1</sup> the temperature referred to was that of the oil bath. This was made clear in the directions given by one of us in a later article.<sup>2</sup> In the latter article it was also stated that a temperature of 135° was sufficient when hydrolysis was carried out in a sealed tube. Consequently the results of Walter Jones<sup>3</sup> essentially agree with those obtained by us.

<sup>1</sup> Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1910, xliii, 3154.

<sup>2</sup> Levene, *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1911, v, 492.

<sup>3</sup> Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93.



# THE NATURE OF THE DIETARY DEFICIENCIES OF THE WHEAT EMBRYO.\*

By E. V. McCOLLUM, NINA SIMMONDS, AND WALTER PITZ.<sup>1</sup>

(From the Laboratory of Agricultural Chemistry of the Wisconsin Experiment Station, Madison.)

(Received for publication, March 25, 1916.)

In former papers from this laboratory we have shown the character of the deficiencies of the wheat kernel<sup>2</sup> and of polished rice<sup>3</sup> respectively as nutrients for growth and reproduction. As a result of this work, together with an extensive experience in observing animals fed with mixtures of purified foodstuffs, we proposed the working hypothesis that in addition to protein, carbohydrate, or fat and mineral elements in suitable amounts and proportions, the diet, in order to support growth or long continued well-being, must contain two factors whose chemical natures are at present unknown. One of these is associated with fats from certain sources, but is not found in the fats of many natural foodstuffs.<sup>4</sup> The other, which is soluble in water and alcohol, is widely distributed in natural foods such as milk, eggs, meats, vegetables, and grains.<sup>3</sup> This factor is apparently never associated with fats. Funk and others have recognized one of these dietary factors, the water-soluble one, in their studies on beri-beri. There is no

\* Published with the permission of the Director of the Wisconsin Experiment Station.

<sup>1</sup> Miss Marguerite Davis participated in a considerable amount of preliminary work directed toward the solution of the problems discussed in this paper. Most of the data accumulated in that work are not included in this discussion, but the information gained therefrom made possible the successful planning of the rations later employed. Credit is due Miss Davis for her part in this investigation.

<sup>2</sup> McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615.

<sup>3</sup> McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181.

<sup>4</sup> McCollum and Davis, *J. Biol. Chem.*, 1913, xv, 167; 1915, xxiii, 231. Osborne, T. B., and Mendel, L. B., *ibid.*, 1913-14, xvi, 424.

doubt that a deficiency of this factor and not the fat-soluble unknown is responsible for the onset of polyneuritis and that the pathological symptoms are entirely relieved by treatment of pigeons with extracts of substances which contain the former but do not furnish the fat-soluble one. McCollum and Kennedy<sup>5</sup> have discussed the desirability of discontinuing the use of the term "vitamine" for these substances which are essential components of an adequate diet, and have suggested the employment of the terms fat-soluble A and water-soluble B instead. These terms are employed in this sense in the present paper.

We deem it of the greatest importance for the purpose of placing human nutrition and animal production upon a sound scientific basis to have, in addition to a full understanding of the essential factors in the diet for growth and the promotion of well-being during maintenance, a thorough knowledge of the causes which are responsible for the failure of nutrition of an animal when confined to a single naturally occurring food substance. We should understand fully why, for example, the corn, wheat, oat, or rice kernel is, individually, totally inadequate to support the life of a grown animal over more than a brief period, or to support growth in the young, as our experience has shown to be the case. Only then shall we be able to proceed to an experimental inquiry into the reasons for the nutritive failure of human beings when eating a liberal variety of foods of vegetable origin, as Goldberger has shown to be the case among pellagrins,<sup>6</sup> or of animals fed certain mixtures with the customary complexity of animal husbandry practice.<sup>7</sup> It is with a view to the acquisition of such knowledge that we are pursuing studies of the character reported in this and the previously cited papers.

The method pursued in our work is based on the following line of reasoning: If a single natural food product fails to nourish an animal adequately, it may be due to: (a) lack of sufficient protein, or to proteins of poor quality; (b) an unsatisfactory mineral content due either to inadequacy of certain elements in amount, or to unsatisfactory proportions among them; (c) an inadequate sup-

<sup>5</sup> McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

<sup>6</sup> Goldberger, J., *J. Am. Med. Assn.*, 1916, lxvi, 471.

<sup>7</sup> Hart, E. B., McCollum E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Experiment Station, Research Bull. 17*, 1911.



ply of the fat-soluble A; (d) of the water-soluble B; (e) or some toxic substance contained therein. One, two, three, four, or all of these factors may operate in inducing nutritive disturbances.

It should be obvious that a systematic procedure in which we feed the substance under investigation supplemented with (a) pure protein only, (b) salt mixture additions only, (c) butter fat only, (d) extracts known to carry the water-soluble B and as little else as is possible, will reveal whether the failure of nutrition involves one factor only, or more than one. If more than one factor is involved, a similar procedure, but with the addition of all possible combinations of pairs of the isolated food ingredients listed above, followed if need be by another series of feeding experiments in which animals are fed the natural foodstuff supplemented with three such uncomplicated additions, in all possible combinations, and if necessary another experiment in which all four additions are made, will give us results which make it possible to consider the components of our rations in an entirely new light. Provided the foodstuffs contain a toxic substance, special procedures will have to be devised for studying its effects.

Similar studies must also be made by this method of procedure, with pairs of the important foodstuffs in varying proportions, the variation of the mixture including sufficient range to reveal the degree to which the deficiencies of the protein mixture of one grain are corrected by the peculiar quantitative relationships among the amino-acids yielded by the proteins of the other grain. The same may be said for the factors other than protein. In this way we shall become able to interpret the biological value of the mixtures of natural foodstuffs which make up the rations which are in common use, in which the attempt is now made to make for safety through variety. We have carried our inquiry into the nature of the dietary deficiencies of several natural products far enough to convince us of the practicability of this method of study. Young rats fed wheat embryo alone never make any growth, although they may live somewhat beyond 4 months (Chart I). When young rats are fed a mixture of 60 parts of wheat embryo and 40 of dextrin, a mixture which furnishes about 20 per cent of protein, very slow growth takes place, and the animals may live for 4 months or more without loss of weight (Chart II). The addition of calcium lactate (2 per cent) to this food mixture will

at any period of their growth suspension induce a noticeable increase in body weight.

We were led to conclude by our experiments on growth in animals fed diets of low protein content, and by metabolism studies with pigs which received all their protein from the wheat embryo, that these proteins have a high biological value. Rats can make an appreciable amount of growth on diets carrying but 4 per cent of wheat embryo proteins,<sup>8</sup> and pigs can retain as much as 39 per cent of the ingested wheat embryo proteins for growth.<sup>9</sup> Since it is not possible when 60 per cent of wheat embryo is employed in the ration to imitate by salt additions the mineral content of any rations which we have fed successfully, because of the high mineral content of the embryo itself, we tried lowering the content of this constituent to give a protein content of 10 per cent in order to be able to make salt additions to the diet with the prospect of rendering the mineral content a favorable one for growth. Lot 521 (Chart III) shows how marked is the improvement of the animals resulting from the correction of the inorganic content of the diet. After 2 to 3 months failure supervenes, accompanied by what appeared to be an infection of the ears, and an edema of the eyes which is frequently seen in rats suffering from an inadequate supply of the fat-soluble A. Ether-extracted wheat embryo gave us much better nutrition than unextracted material. The cause of this will appear later.

That the failure of nutrition of the rats in Lot 521 (Chart III) was due to a lack of the fat-soluble A is made apparent by the performance of Lot 397 (Chart IV) whose ration was identical with that of Lot 521 except that 5 per cent of butter fat replaced an equivalent amount of dextrin. These rats grew to normal size more rapidly than the curve of normal expectation led us to anticipate and the two females each produced two litters of young. None of these young were reared, however. This and the fact that the three rats in this group all died after 9 and 10 months, led us to suspect that the ration still possessed certain unfavorable factors in some degree. The low protein content may be sufficient to explain this, but the history of Lot 508 (Chart

<sup>8</sup> McCollum and Davis, *J. Biol. Chem.*, 1915, xx, 415.

<sup>9</sup> McCollum, E. V. See Henry, W. A., and Morrison, F. B., *Feeds and Feeding*, Madison, 15th edition, 1915. 80.

V) points to some other explanation as more probable. It is interesting to note that if Ration 397 is fed without the salt mixture addition it is incapable of inducing any growth whatever (see Chart VIII, Lot 585). The specific reasons for this we shall discuss later in connection with another investigation.

Lot 508 (Chart V) received the same ration as Lot 397 (Chart IV), except that 5 per cent of dextrin was replaced by 5 per cent of purified casein. The growth of these animals has been normal, but two females have each failed to keep their two litters of young alive beyond the first 3 days of life. We were, therefore, led to conclude that there is a slight degree of toxicity in this ration. The protein factor in Lot 397 is therefore not the only factor involved in preventing repetition of reproduction and the successful rearing of the young.

That there is in reality a certain degree of toxic effect of wheat embryo and that this resides in the oil is clearly shown by the records of Lot 495 (Chart VI) whose ration differed from Lot 397 only in that the wheat embryo was not extracted with ether. This group made about half normal growth during 3 to 4 months, as contrasted with a somewhat more rapid increase in weight by Lot 397 than our normal curve predicts. All the animals in Lot 495 became emaciated and presented a very miserable appearance.

The ration of Lot 415 (Chart VII) differed from the preceding one in having 10 per cent more wheat embryo (43 per cent of the food mixture). Although the protein content of this ration was raised to 13 per cent by this modification and was 25 per cent greater than in Lots 397 and 495 (Charts V and VI), there was but slight improvement in the condition of the rats. They presented the same general symptoms as did Lot 495. The removal of the fats by extraction with ether removes in great measure the toxicity of the embryo. We are at present conducting experiments to determine whether the toxicity of these fats resides in the fatty acids themselves or is due to some substance soluble in the fats and in ether.

The marked difference between the nutritive value of the ration of Lot 508 (Chart V) with and without the butter fat addition is illustrated by comparing this group with Lot 566 (Chart IX) which received no butter fat. There was a rapid increase in

weight during the first 4 weeks in some of these rats, but complete failure of nutrition was never deferred much beyond 70 days, and in some cases it took place much earlier. The fat-soluble A must be added to wheat embryo, regardless of what other additions or modifications may be made with diets of the character we are here considering before normal nutrition can be attained. This does not imply the absence of this factor from the wheat embryo; indeed McCollum and Davis<sup>10</sup> have demonstrated that the latter does contain it, and this is further supported by the records of Lot 498 (Chart X). This ration will not induce growth without the addition of the fat-soluble A. 5 per cent of ether-extracted wheat embryo carries enough of this substance to induce growth for 90 days in a manner similar to that induced by carefully purified protein, carbohydrate, and salts, with a content of 20 per cent of milk sugar of ordinary laboratory purity. Such lactose carries a sufficient amount of both the fat-soluble A and the water-soluble B to induce growth in vigorous individuals at but little below the average normal rate, over periods of 60 to 100 days.<sup>11</sup> This result reveals the unexpected fact that in removing the fats from wheat embryo by extraction with ether the fat-soluble A is not wholly extracted. Lot 509 (Chart XI) further illustrates this fact. This ration, without the factor in question, does not support growth. Both the unknown A and B are supplied by 5 per cent of fat-free germ, in amounts sufficient to induce good growth over several months. It is this tendency of the fat-soluble A to remain associated with lactose, together with the generous amount of the water-soluble B contained in milk serum which determines the growth-promoting power of Osborne and Mendel's protein-free milk. Provided these factors are added, success can be attained in nourishing growing animals with salt mixtures prepared from purified reagents. Since we have shown how the water-soluble B can be obtained from various sources by the use of several solvents, it is now easy to conduct experiments to compare the relative values of purified proteins under experimental conditions much more satisfactory than to employ purified proteins in rations containing 6.9 per cent of their total nitrogen in unknown forms, as was done by Osborne and Mendel

<sup>10</sup> McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 179.

<sup>11</sup> McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 231.

in all cases where the diets contained 18 per cent of protein and 28.3 per cent of protein-free milk which contained 0.76 per cent of N. The desirability of improving the technique of this line of investigation is emphasized by our observations that the nitrogen of protein-free milk appears to possess considerable value as a source of protein cleavage products.<sup>3</sup>

That it is not lack of fats which caused the nutritive depression in Lot 566 (Chart IX) is made apparent by the records of Lot 523 (Chart XII). These rats were given a ration precisely like Lot 508 (Chart V) except that 5 per cent of corn oil replaced 5 per cent of butter fat. During 98 days these animals were markedly depressed in growth, the depression manifesting itself only after 30 days. After 98 days butter fat was added to the diet, with the result that growth was at once resumed. The marked inferiority of corn oil with respect to its content of the fat-soluble A as compared with an equivalent amount of butter fat is emphasized by these results. That corn oil is not entirely without this growth-promoting factor is indicated by unpublished results of this laboratory.

Although in Lot 397 (Chart IV) 10 per cent of wheat embryo proteins sufficed for normal growth and supported females while they produced two litters of young (but without rearing the young), and the ration was not improved so far as the rearing of the young was concerned by the addition of 5 per cent of casein (Lot 508, Chart V), the food mixture is rendered more satisfactory for resistance to unfavorable factors which may operate in the diet. Thus in Lot 520 (Chart XIII) the ration resembled that of Lot 523 (Chart XII) in all respects except that the 5 per cent of casein of the latter's ration was replaced by dextrin. These animals receiving corn oil as their only source of fat did not make as good growth without the casein added (Lot 520) as did Lot 523.

This case illustrates a principle in nutrition which has not hitherto been fully appreciated; *viz.*, that a single factor (protein) in a ration may appear to admit of the maximum performance of the animal with respect to growth, without itself representing the optimum amount or character. When this circumstance prevails, it may entirely escape notice, yet if in another ration exactly like it except that a second factor tends to injure the animal, nutritive failure may result. In such a case as the latter, the im-

provement of the protein factor by the addition of more protein or by the substitution of a better protein, the plane of protein intake remaining unchanged, the animal may make the maximum performance notwithstanding the unfavorable character of the injurious factor of the ration.

For example, Lot 397 (Chart IV) contains but 10 per cent of protein, all furnished by ether-extracted wheat embryo. This suffices to support growth at the maximum rate. In Lot 495 (Chart VI), whose ration was exactly similar except that the wheat embryo carried the toxic wheat embryo fats, marked depression of growth was observed. Lot 524 (Chart XIV) received the same ration as did Lot 495, but with 5 per cent more protein in the form of casein, replacing dextrin, and in spite of the toxic effect of the embryo fats growth was normal for a period of over 4 months and three females produced six litters of young during that period, although none of these were reared. This additional 5 per cent of casein caused Lot 524 to overcome the toxic action of the fats in a considerable degree. Lot 405 (Chart XV), whose ration differed from Lot 524 only in having but 2 per cent of butter fat while the latter had 5 per cent, illustrates the same principle. With the low butter fat content the casein content like that of Ration 524 did not protect the animals from the stunting effect of the toxic fats. The 5 per cent of casein together with the higher content of butter fat served to make the rats of Lot 524 more resistant to this unfavorable factor. Such results suggest a plausible explanation for the marked difference in the resistance to poisoning with acetonitrile, shown by rats and mice, which resulted in the experiments of Hunt,<sup>12</sup> through the influence of the diet. We have elsewhere called attention to this same principle in its operation in determining whether the inorganic content of the diet, when of unsatisfactory make-up, will produce noticeable depression in the growth and health of the experimental animals.<sup>13</sup>

Further illustration of the toxic effect of wheat embryo fats is seen in Lot 517 (Chart XVI) whose ration contained but a trace of fats from this source, and Lot 448 (Chart XVII) whose ration was exactly similar except that the 6 per cent of dextrin in the former was replaced by 6 per cent of wheat embryo fats in

<sup>12</sup> Hunt, R., *Bull. Hyg. Lab., U. S. P. H.*, 69, 1910.

<sup>13</sup> McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 641.

the latter. The growth curves of Lot 448 are depressed in an easily noticeable degree, and a tendency to loss of hair in spots further points to the unfavorable effects of this addition.

The question naturally arises on inspecting the data presented in this paper, when in Lots 498 and 509 (Charts X and XI) 5 per cent of ether-extracted wheat embryo served to furnish enough of the fat-soluble A to induce nearly normal growth during a period of 3 to 4 months, why does not a ration carrying 33 per cent of wheat embryo supply an amount of this substance which meets all the needs of the animals? In all such rations we see failure without butter fat (Lots 520, 521, and 523) and pronounced benefit from its addition to or inclusion in the diet (compare Lot 521, Chart III, with Lot 397, Chart IV; Lot 566, Chart IX, with Lot 508, Chart V; Lot 405, Chart XV, with Lot 524, Chart XIV). This question seems to admit of but one explanation in the light of the data available, and this we have adopted tentatively: Wheat embryo is not entirely freed from its toxicity even by long continued extraction with ether (20 hours). When therefore the extracted material is added in progressively greater amounts in a series of rations two factors are affected which operate in opposition to each other physiologically. When a low plane of fat-free embryo is employed a small amount of fat-soluble A is introduced, and but little of the toxic substance. The amount of the fat-soluble A is not great enough to support young rats from infancy through complete growth, and failure ultimately supervenes. The toxic factor may even here operate in some degree to bring about nutritive failure, through a cumulative effect. When higher planes of ether-extracted wheat embryo are employed, the injurious effects of its toxicity tend to counterbalance the beneficial effect of the added fat-soluble A, and the animal is prevented from developing normally.

This hypothesis is in some measure supported by the behavior of Lot 463 (Chart XVIII) whose ration consisted of ether-extracted wheat embryo 60.0, salts (314) 5.0, and dextrin 35.0 per cent. These rats grew at about one-third the normal rate during the first 100 days. Thereafter two of the four made a decided response in growth when butter fat was added to the diet. It is recognized that the mineral content of this ration is not satisfactory for growth when any other factors are unfavorable.

We present also the records of Lot 463-A (Chart XIX) whose ration consisted, aside from its content of 2 per cent of wheat embryo, of substances free from the water-soluble B. Even this small amount of embryo supplied this factor in quantity sufficient to promote growth and well-being over a long period. The content of water-soluble B in this ration is doubtless near the minimum on which nutrition can remain nearly normal, and it is highly probable that the resistance of these rats to unfavorable agencies of any character would be heightened by a higher content of the water-soluble B in the diet.

In former papers from this laboratory we have pointed out the distinctly injurious effects resulting from feeding rations too closely restricted to the wheat plant and its parts.<sup>7,14</sup> The necessary modifications of the wheat kernel which led to successful nutrition with rations consisting principally of the wheat kernel have been described by McCollum and Davis.<sup>2</sup> In the present discussion of the special properties of wheat embryo we are able to point out definitely that the toxic factor is in the fat fraction. There is a possibility that the failure to attain perfect nutrition with rations consisting of fat-free wheat embryo supplemented with isolated food substances such as protein, salts, and butter fat is to be explained as the result of the failure of ether to remove entirely the toxic substance.

#### SUMMARY OF RESULTS.

The experiments reported in this paper reveal the fact that the wheat embryo contains qualitatively all the factors essential for the promotion of growth and well-being in an animal, but these are not so proportioned that it can serve as a satisfactory diet without several modifications.

The mineral content must be modified in certain respects before growth can proceed at all.

The character of the proteins is excellent; no other proteins from plant sources which we have studied are superior to them. Rations containing but 10 per cent of these proteins are wholly adequate for growth at the maximum rate.

Both the fat-soluble A and the water-soluble B, the factors which must be supplied by an adequate diet, but whose chemical

<sup>14</sup> Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373



natures are as yet unknown, are present; the first, in moderate concentration; the second, in very high concentration as measured by the needs of the growing animal. 2 per cent of wheat embryo supplies enough of the water-soluble B to promote growth at the normal rate for several months.

There is contained in the wheat germ a substance which is distinctly toxic to animals. This is in great measure removed by extraction with ether, and is found in the fat fraction. We have not yet determined whether the toxicity is due to peculiarities in the chemical nature of the fats themselves or to something which is associated with the fats.

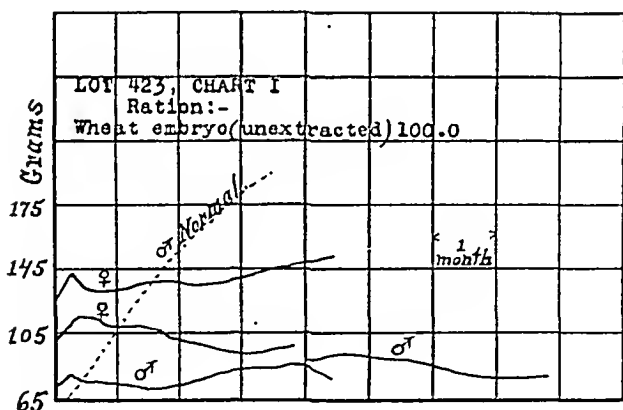


CHART I. Lot 423. These records show the failure of young rats to grow when limited to wheat embryo as their sole source of nutriment. The wheat embryo employed in these experiments contained 30.0 per cent of protein.

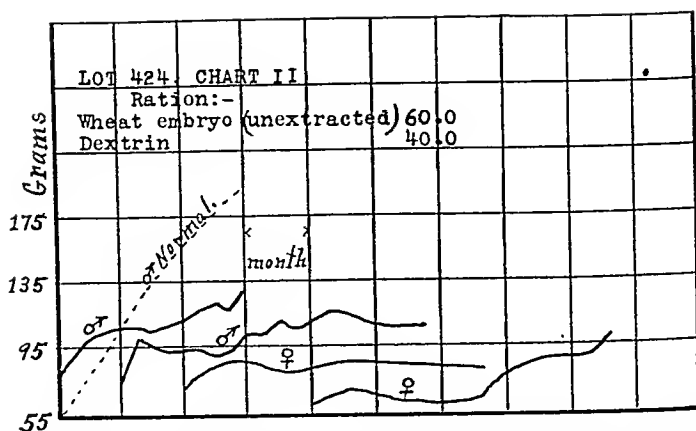


CHART II. Lot 424. Shows a marked improvement in young rats fed wheat embryo 60 and dextrin 40 per cent. With this food mixture slow but long continued growth can take place (compare Chart I).

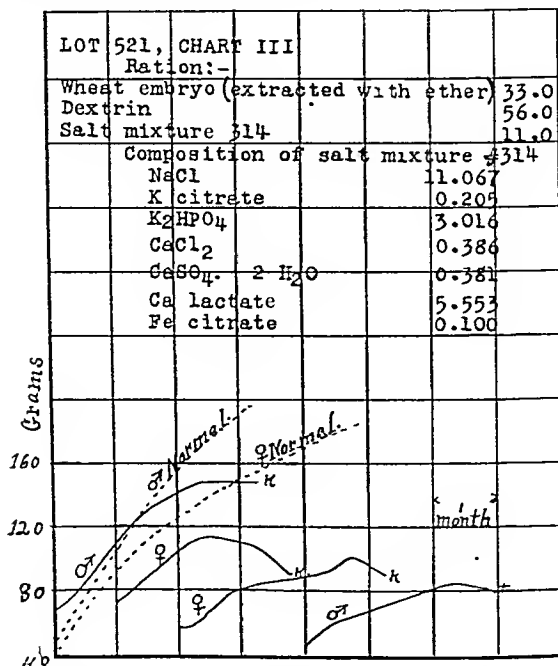


CHART III. Lot 521. Illustrates the marked improvement in the growth of young rats fed ether-extracted wheat embryo to supply 10 per cent of protein, together with dextrin and a salt mixture. While failure of nutrition supervenes after 3 or 4 months on this ration, the addition of butter fat renders it surprisingly efficient in promoting growth and sustained well-being (compare with Lot 397, Chart IV).

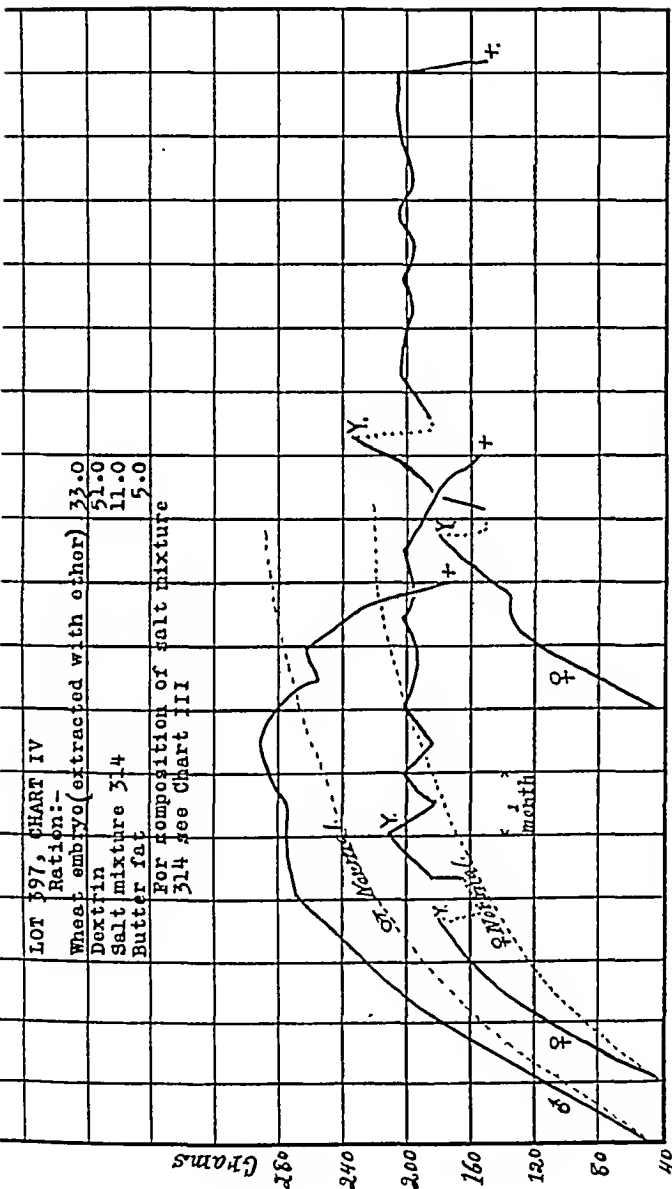


CHART IV. Lot 397. Without the butter fat contained in the food mixture supplied to these rats, depressed growth and early failure supervenes (Lot 521, Chart III). This furnishes the fat-soluble A, a substance of unknown chemical nature which is indispensable for growth.

Wheat embryo fats contain something which is distinctly toxic to animals. This ration if made up with embryo from which the fats have not been removed is distinctly inferior to the above where fat-free embryo was employed (compare Lot 495, Chart VI).

Without the salt mixture additions this ration does not induce any growth (see Lot 585, Chart VIII).

The failure of these rats to produce more than two litters of young each, and their inability to nourish those which they produced, points to the existence of a toxic factor in the wheat embryo after it has been thoroughly extracted with ether. In this and succeeding charts Y marks the birth of young.

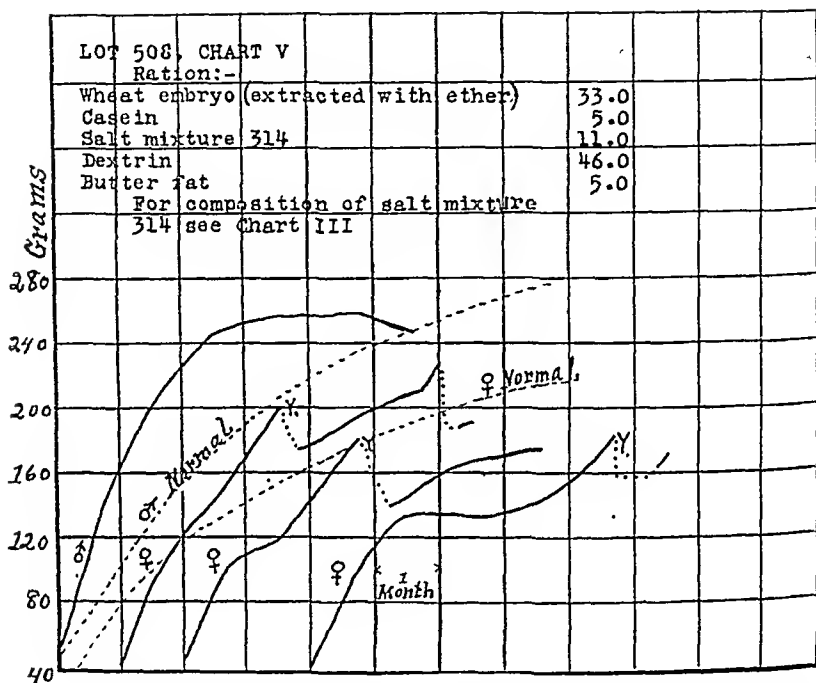


CHART V. Lot 508. This group received a ration similar to Lot 397 (Chart IV), except that 5 per cent of casein replaced an equivalent amount of dextrin. Its protein content was therefore about 15 per cent as compared with 10 per cent in Lot 397. This modification of the diet was employed in order to determine whether the failure of the rats in Lot 397 to rear their young was the result of the low protein content of the food mixture. Since four litters of young produced by Lot 508 have all been allowed to die within 3 days, it appears that the low protein content supplied to Lot 397 was not entirely responsible for the loss of their young. It seems evident that the wheat embryo is slightly toxic even after extraction with ether, although this solvent takes out something which is distinctly injurious.

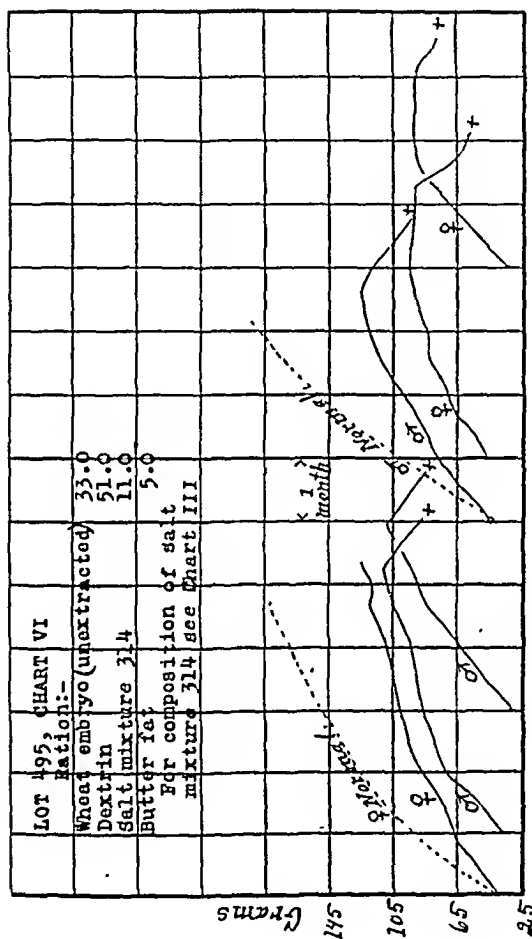


CHART VI. Lot 495. These records show the great inferiority of wheat embryo containing its fat content to that which has been extracted with ether (see Lots 397 and 508, Charts IV and V). The ration here employed differed from that of Lot 397 only in respect to its content of wheat embryo fats. About 3.3 per cent of these fats were supplied by this ration and the rate of growth was depressed about one-half by their presence.

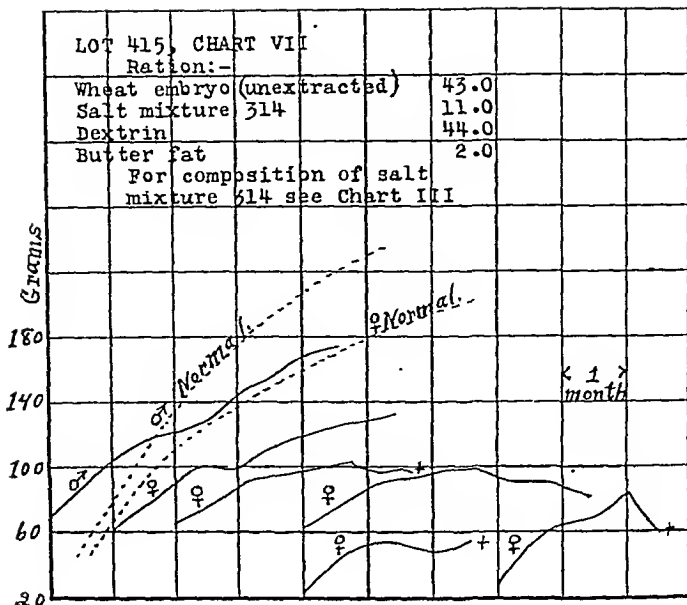


CHART VII. Lot 415. These records again illustrate the injurious effects of the wheat embryo fats, on growing rats. This ration made up with ether-extracted wheat embryo would be greatly superior to the formula here given (compare Lots 397 and 508, Charts IV and V).

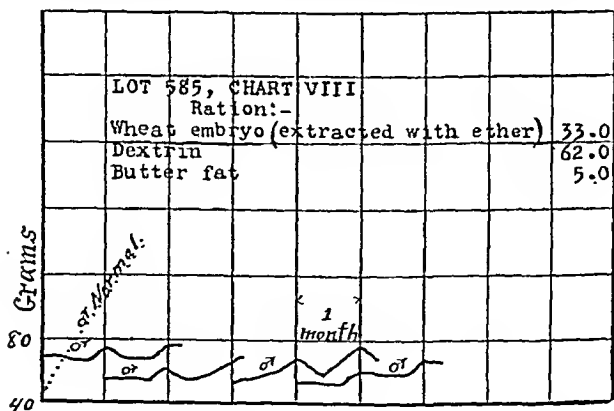


CHART VIII. Lot 585. The curves of these rats illustrate the great importance of the proper adjustment of the mineral content of the diet. The animals in Chart VIII received a ration like that of Lot 397 (Chart IV), except that no salt addition was made, dextrin being added in place of the salt mixture. In this instance we have an example of a ration in which the adjustment of the inorganic content determines whether there shall be complete suspension of growth (without salt additions), or growth at a rate exceeding the normal expectation, as illustrated by Lot 397 (Chart IV).

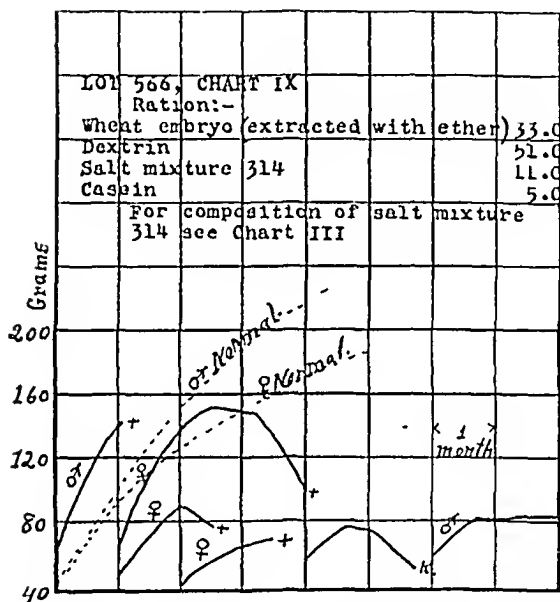


CHART IX. Lot 566. This chart illustrates the great benefit to the animals of including butter fat, or some fat of the group which possesses the same growth-promoting property, in diets where the fat-soluble  $\text{A}$  is supplied by wheat embryo. Although this factor is present, a higher content makes the difference between almost complete failure and nearly complete success in the nutrition of growing animals (compare with Lots 397 and 508, Charts IV and V)





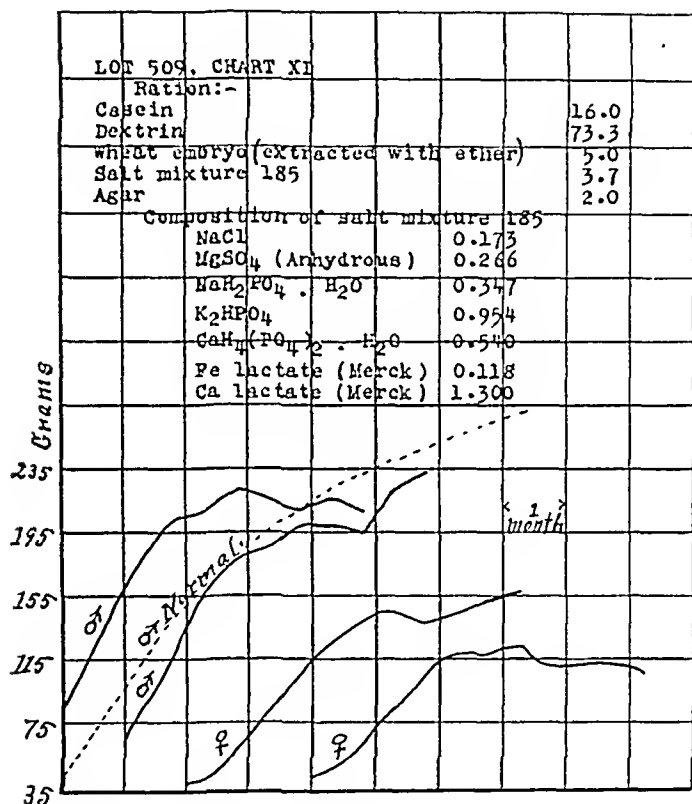


CHART XI. Lot 509. Further illustrates the presence of the fat-soluble A in wheat embryo. Without this factor there would be no growth with this ration. Both the fat-soluble A and water-soluble B are furnished in this ration by 5 per cent of wheat embryo which has been freed from fats by extraction with ether (compare with Lot 498, Chart X, and with Lots 523 and 520, Charts XII and XIII).

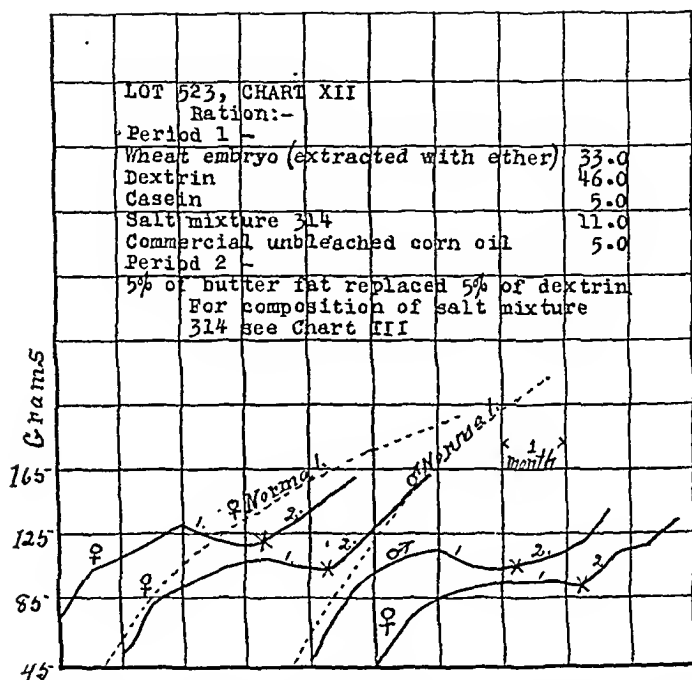


CHART XII. Lot 523. That the failure of the rats to grow on Ration 521 (Chart III) was not due to lack of fats is shown by the records here presented. Lot 523 received a ration closely similar to Lot 508 (Chart V) except that here the butter fat was replaced by 5 per cent of corn oil. The inferiority of these rats as compared with those of Lot 508 which received 5 per cent of butter fat is unmistakable (compare Lot 508, Chart V, and Lot 520, Chart XIII). During the last month covered by these curves 5 per cent of butter fat was included in the ration, and this resulted in a marked response with growth. Corn oil is not the equivalent of butter fat in supplying the fat-soluble A (compare Lot 523 with Lot 508, Chart V, and Lot 397, Chart IV with Lot 520, Chart XIII).

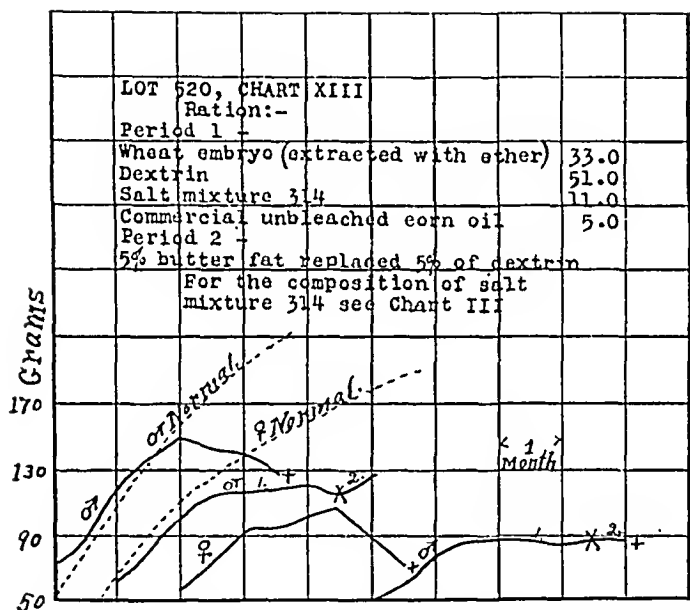


CHART XIII. Lot 520. These curves again point to the fact that corn oil is not equivalent to butter fat in its growth-promoting power. The fat-soluble A is present in the corn kernel, but in relatively low amount (compare Lot 397, Chart IV, and Lot 523, Chart XII).

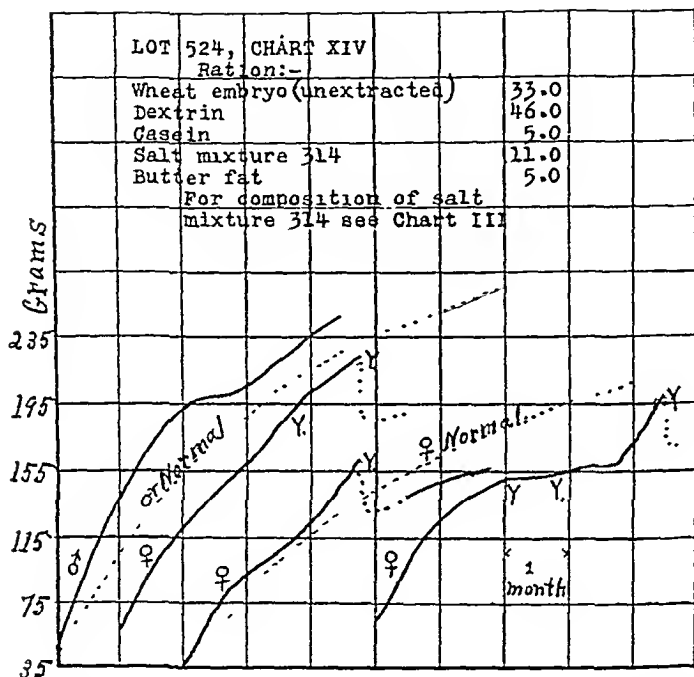


CHART XIV Lot 524. Although 10 per cent of wheat embryo proteins are able to support growth at the maximum rate (Lot 397, Chart IV), the addition of 5 per cent of casein, as in the ration of Lot 524, enables the animals to withstand to a marked degree the injurious effects of wheat embryo fats (compare Lot 495, Chart VI). The ration of Lot 495 differed from that of Lot 524 only in having 5 per cent of casein replacing dextrin. These records illustrate how an unfavorable factor of any character in the diet may escape notice if all other factors are made favorable. With the protein content less favorable (Lot 495, Chart VI), or with the fat-soluble A reduced to nearly the minimum requirement (Lots 523 and 520, Charts XII and XIII), the toxic effects of the wheat embryo fats manifest themselves.

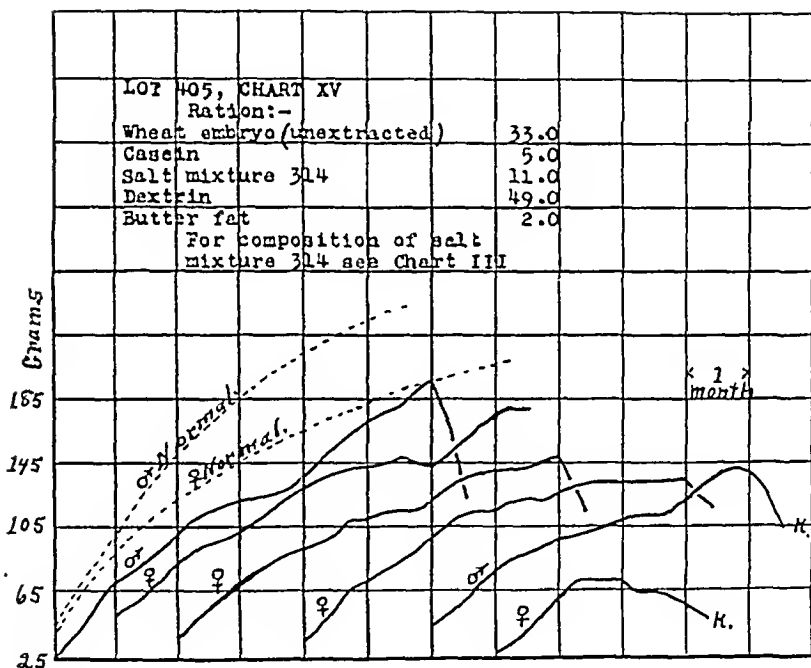


CHART XV. Lot 405. Illustrates the distinct advantage to the growing young of having an abundance of the fat-soluble A in the diet. The curves of Lot 405 which received 2 per cent of butter fat are decidedly inferior to those of Lot 524 (Chart XIV), whose ration differed only in having 5 per cent of butter fat.

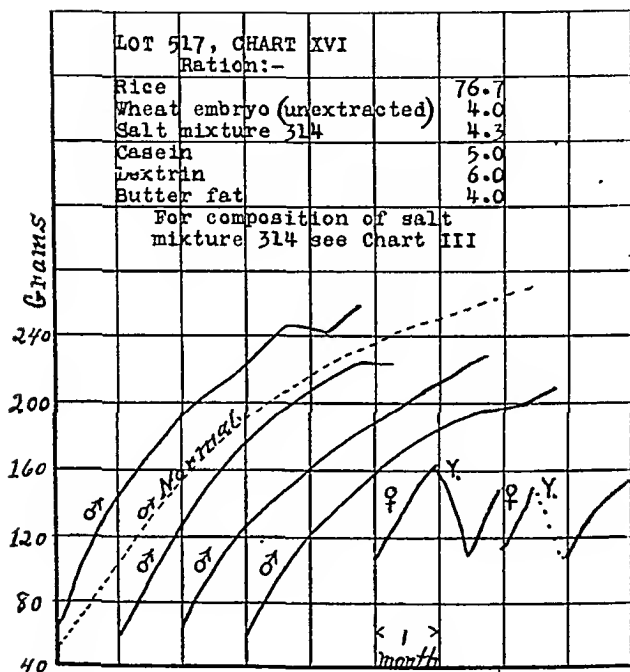


CHART XVI. Lot 517. Showing the records of rats which received a ration containing but a trace of wheat embryo fats, as compared with Lot 448 (Chart XVII), whose ration was closely similar but contained 6 per cent of wheat embryo fats replacing 6 per cent of dextrin. The depressing effect of the wheat embryo fats is readily seen (compare Lot 448, Chart XVII).

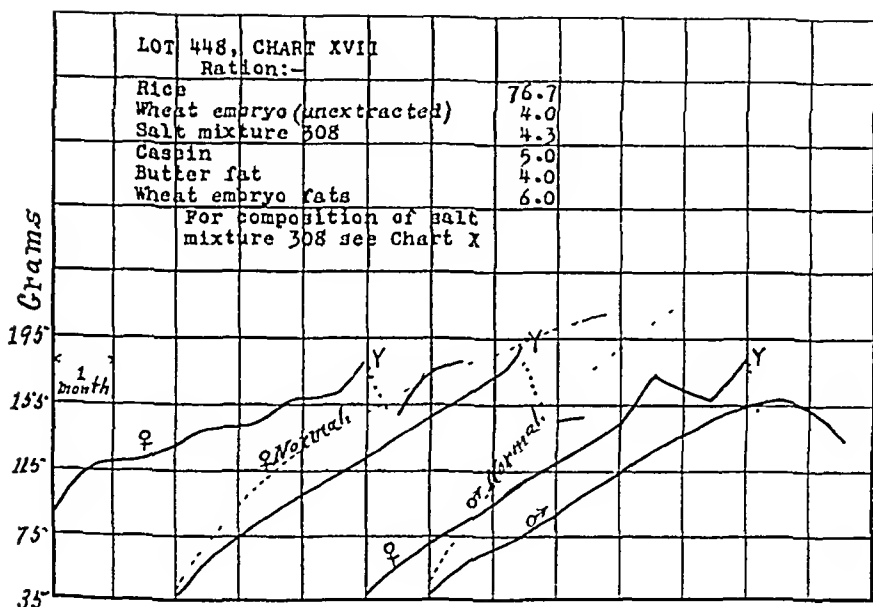


CHART XVII Lot 448 These records show the toxic effect of 6 per cent of wheat embryo fats in the diet of these rats. Their growth was distinctly below the normal rate, and they were inferior to those of Lot 517 (Chart XVI), whose ration was similar except that 6 per cent of dextrin replaced the wheat embryo fats of Lot 448 (compare Lot 517, Chart XVI)

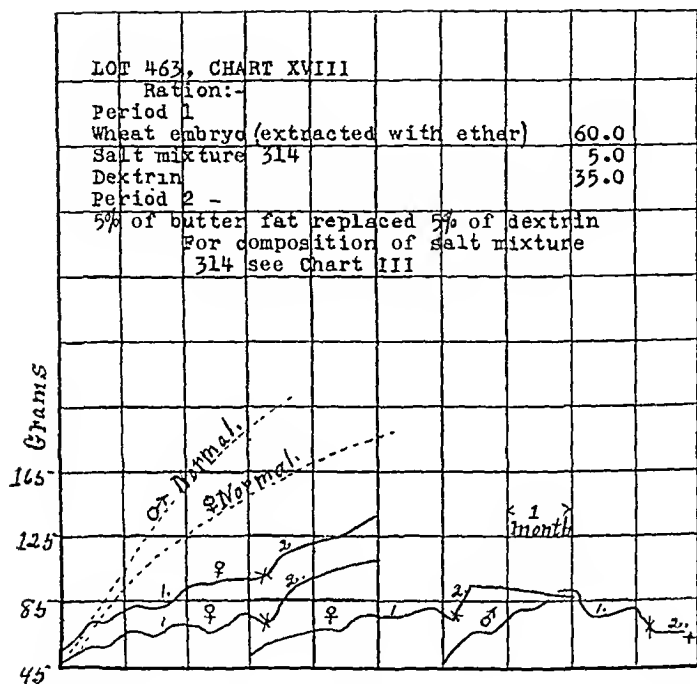


CHART XVIII. Lot 463. While 5 per cent of ether-extracted wheat embryo furnished enough of the fat-soluble A in Lot 498 (Chart X) and Lot 509 (Chart XI) to induce good growth over a period of 3 months or more, animals whose diets contained liberal amounts of ether-extracted embryo (33 per cent or higher) still responded with better growth and improved well-being to the addition of butter fat, which supplies this factor. This we can explain only by assuming that the fat-free embryo still contains something toxic, and that by making all factors except this toxicity as nearly ideal as possible the resistance to the toxic effect is heightened. Lot 463 contained twelve times as much of the fat-soluble A as did Ration 498 or 509, yet growth was depressed and after 100 days the rats still responded in two cases to a butter fat addition. The injurious effect of the toxic factor runs counter to the beneficial effects of the higher level of the fat-soluble A.



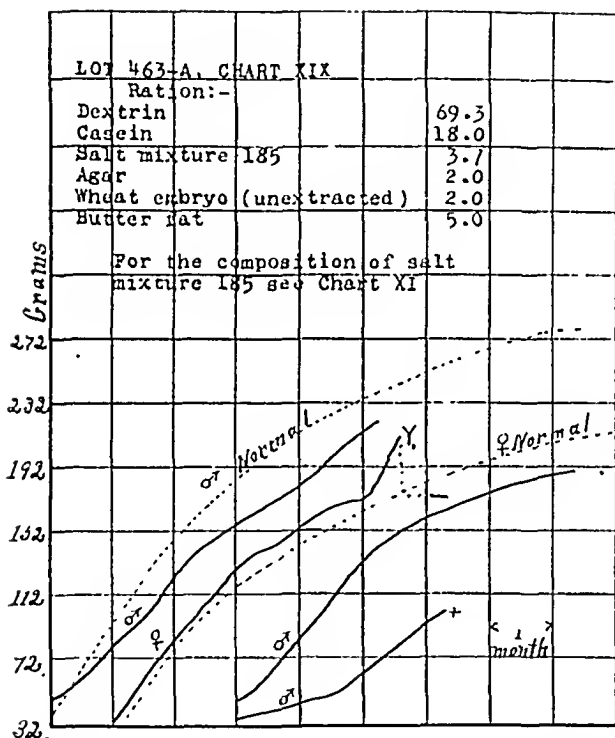
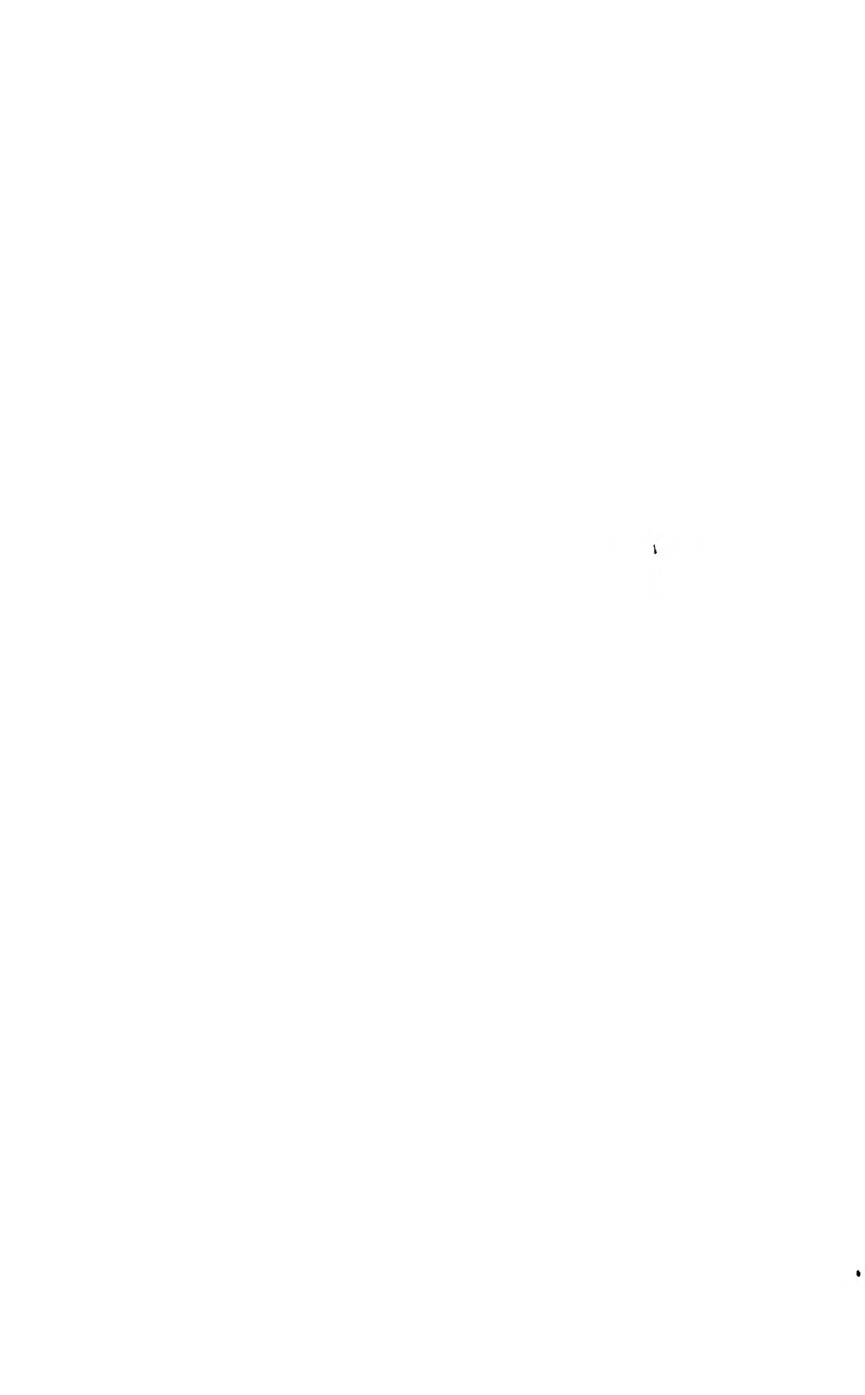


CHART XIX. Lot 463-A. These records illustrate the high content of the water-soluble B in wheat embryo. Without this factor in the diet this ration will not support growth. As little as 2 per cent of wheat embryo furnishes enough of this substance to promote growth over a long period, but is probably somewhat below the optimum.



# THE SEPARATION OF AUTOGENOUS AND ADDED HYDROCYANIC ACID FROM CERTAIN PLANT TISSUES AND ITS DISAPPEARANCE DURING MACERATION.\*

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Hydrocyanic acid is supposed to occur in plants only in combination. It has been identified in glucosides. In plant tissues, therefore, the hydrocyanic acid in such a compound as a glucoside must first be set free by hydrolysis before this acid can be determined. The hydrolysis has usually been effected either by treating the plant tissue with fixed acid, or by macerating the material in water alone, or in water in presence of added emulsin or other hydrolyzing enzyme. When hydrolysis is performed by fixed acids, hydrolysis and distillation are combined in one operation by distilling with dilute acid. If maceration either with or without added enzyme causes hydrolysis, the hydrocyanic acid liberated is subsequently distilled. In either case the hydrocyanic acid in the distillate is estimated by some of the well known methods.

No systematic study of all factors involved in the separation of hydrocyanic acid from plant tissues, such as is customary in the study of analytical methods, has been recorded in the literature. For example, the possibility of loss of hydrocyanic acid during maceration has not been investigated, although there are statements in the literature indicating that such losses occur. Thus, in 1912 Dezani<sup>1</sup> published an investigation showing that cyanide

\* The essential facts included in this paper were presented before the Biological Chemistry Section of the American Chemical Society, in session December 27-30, 1911, at Washington, D. C.

<sup>1</sup> Dezani, S., La riduzione dell' acido cianidrico ad opera dei succhi delle piante, *Atti II Cong. naz. chim. appl.*, Turin, 1911, 368.

added to plant juices expressed under a pressure of 300 atmospheres is decomposed with the formation of ammonia. The work reported here by us was essentially completed before Dezan's publication. Further, Waller<sup>2</sup> finds that in the presence of indifferent narcotics, such as ether, chloroform, or alcohol, the rate of evolution of hydrocyanic acid from the leaves of the cherry laurel, *Prunus laurocerasus*, is much less rapid at 16–18°C. than at 40°C. In water at room temperatures practically none is set free. It has usually been assumed that, in distilling in presence of small amounts of the fixed acid, hydrolysis is rapid. Dunstan and Henry<sup>3</sup> have claimed that lotusin, the cyanogenetic glucoside of *Lotus arabicus*, is less rapidly hydrolyzed by sulfuric acid than by hydrochloric acid. Wirth<sup>4</sup> has shown that in distilling the cherry laurel water of the Dutch Pharmacopeia, hydrocyanic acid and aldehyde distill over uncombined, combining, however, in the distillate until a certain equilibrium is reached. Rocques and Lévy<sup>5</sup> have found that in old cherry brandies hydrocyanic acid occurs in combination and is evolved only gradually during distillation. Furthermore, after the conclusion of the experiments recorded in the present paper, De Jong<sup>6</sup> published the observation that the enzyme of *Pangium edule* decomposes the cyanogenetic glucoside, gynocardin, forming both a diketone and hydrocyanic acid, the latter in part disappearing as such because it combines with the diketone. Henry and Auld<sup>7</sup> have shown that to a slight extent sucrose, levulose, and ammonium sulfate retard the rate at which hydrocyanic acid is set free from phaseolunatin by the

<sup>2</sup> Waller, A. D., A new method for the quantitative estimation of hydrocyanic acid in vegetable and animal tissues, *Proc. Roy. Soc., Series B*, 1909–10, LVIII, 574

<sup>3</sup> Dunstan, W. R., and Henry, T. A., The nature and origin of the poison of *Lotus Arabicus*, *Phil. Tr. Roy. Soc., Series B*, 1901, xciv, 515.

<sup>4</sup> Wirth, P. H., Untersuchungen über Blausäure-Benzaldehydösungen in Verbindung mit Kirschchlorbeerrwasser, *Pharm. Weekblad*, 1911, XLIII, 1049; cited from *Arch. Pharm.*, 1911, cxlii, 382

<sup>5</sup> Rocques, X., and Lévy, L., Sur la nature des composés cyanés des kirschs, *Compt. rend. Acad.*, 1909, cxlviii, 494

<sup>6</sup> De Jong, A. W. K., La décomposition de la gynocardine par l'enzyme des feuilles de *Pangium edule*, *Rec. trav. chim. Pays-Bas*, 1911, XXX, 220

<sup>7</sup> Henry, T. A., and Auld, S. J. M., The occurrence of cyanogenetic glucosides in feeding-stuffs, *J. Soc. Chem. Ind.*, 1908, XXVII, 428.

enzyme which accompanies this glucoside in a variety of the lima bean, *Phaseolus lunatus*. Glucose present in small quantities markedly retards the rate at which hydrocyanic acid is set free, while the action of invert sugar is less marked. Apparently, this was ascertained by determining the amount of hydrocyanic acid set free under each set of conditions. The amount of hydrocyanic acid remaining combined seems not to have been determined. Henry and Auld<sup>7</sup> devised a method for the determination of hydrocyanic acid in plant tissues by which the cyanogenetic glucoside is partially separated from the plant tissue by hot alcohol before it is hydrolyzed.

It is, therefore, evident from the literature that the determination of hydrocyanic acid present in plant tissue is a matter of some difficulty. The hydrolysis by means of acid is not applicable in all cases since certain cyanogenetic glucosides, such as amygdalin, under the usual conditions of distillation with acids yield only a small percentage of hydrocyanic acid.<sup>8</sup> The determination of hydrocyanic acid by means of an enzyme may be inaccurate, as pointed out by Henry and Auld, because of the presence of substances such as glucose. Henry and Auld assumed that such substances retard the decomposition of the glucoside by the enzyme. However, they do not state that this phenomenon might not possibly have been due to the combination of the liberated hydrocyanic acid with some constituent of the medium, or to the destruction of the hydrocyanic acid, or to its change in such a way that it is not recoverable during distillation. The possibility that the hydrocyanic acid set free is at once combined or destroyed seems worthy of investigation, especially when the determination is made by macerating plant tissue before distilling. Under these conditions it is probable that the protoplasm of a macerated plant remains active for some time. It is easily possible that this protoplasm retains the power to metabolize the liberated hydrocyanic acid exactly as does the normal plant.

An investigation of the influence of duration of distillation on the yield of hydrocyanic acid from plant tissue and the possibility of the disappearance of hydrocyanic acid during maceration is presented in this paper.

<sup>8</sup> Ludwig, H., Einwirkung verdünnter Säuren auf eine Reihe von Bitterstoffen, *Arch. Pharm.*, 1855, cxxvii, 138.

For this study the cyanogenetic plants *Prunus virginiana* or the common wild or choke cherry, *Andropogon sorghum* or the ordinary sorghum, and the grass *Panicularia nervata*<sup>9</sup> were gathered in the District of Columbia or in Arlington, Virginia, in August and September of 1911 and 1912. For use in control experiments *Sambucus canadensis*, or the common elder, was gathered in 1912. All experiments were performed within 6 hours of the time of collection of the plants. Only the leaves of these plants were distilled from 5 per cent sulfuric acid. The distillate was received in an excess of potassium hydrate. Hydrocyanic acid in the alkaline distillate was determined according to the method devised by Berl and Delpy<sup>10</sup> in which the hydrocyanic acid is converted into Prussian blue and the latter determined colorimetrically.

100 gm. portions of the unmacerated leaves of *Andropogon sorghum*, of *Panicularia nervata*, and of *Prunus virginiana*, and 100 gm. portions of the macerated leaves of *Prunus virginiana* were separately distilled in 5 per cent sulfuric acid, the distillate at the end of each half hour being tested to learn when hydrocyanic acid ceases to be evolved. At a uniform, slow rate of distillation it was found that at the end of the first hour of distilling all of the hydrocyanic acid from *Andropogon* and *Panicularia* had been evolved. It required, however, 4 hours to distill all of the hydrocyanic acid from *Prunus*. This was true of the portions containing the macerated leaves as well as of those containing the unmacerated leaves. Even increasing the rate of distilling to violent boiling did not increase the rate at which hydrocyanic acid was set free.

Experiments were also made which show the effect of maceration on the amount of hydrocyanic acid obtainable from *Prunus virginiana*. Other experiments were made to show the effect of maceration on the amount of hydrocyanic acid recoverable from *Andropogon sorghum* and from *Sambucus canadensis* to which in some cases potassium cyanide alone had been added and to which

<sup>9</sup> Alsberg, C. L., and Black, O. F., Concerning the distribution of cyanogen in grasses, especially in the genera *Panicularia* or *Glyceria* and *Tridens* or *Sieglingia*, *J. Biol. Chem.*, 1915, xxi, 601.

<sup>10</sup> Berl, E., and Delpy, M., Über die quantitative colorimetrische Bestimmungen kleiner Blausäure-Mengen, *Ber. chem. Ges.*, 1910, xliii, 1430.

in others potassium cyanide and glucose had been added. The experiments with *Prunus virginiana* and *Andropogon sorghum* are recorded in the table.

TABLE I.

*The Quantities of Hydrocyanic Acid Recoverable from the Leaves of Prunus virginiana and of Andropogon sorghum Macerated with Potassium Cyanide.*

No.	Plant.	Weight of leaves.	Volume of 5 per cent H <sub>2</sub> SO <sub>4</sub> .	Autogenous HCN in leaves estimated as KCN	Weight of KCN added	Maceration		HCN as KCN.		
						Time	Temperature.	Calculated.	Found.	
		gm.	cc.	gm.	gm.	hrs.	°C.	gm.	gm.	per cent
A.										
1	<i>Prunus virginiana</i> .	50	350		0 0000	0			0 0345	
2	" "	50	350		0 0000	24	Room	0 0545	0 0390	71
B.										
3	<i>Andropogon sorghum</i> .	50	300		0 0000	0			0 0231	
4	" "	50	300	0 0231	0 1150	100	Room.	0.1334	0 0931	67
5	" "	50	300	0 0234	0 0575	6	37	0 0509	0 0481	59
6	" "	50	300	0 0234	0 1150	24	37	0 1334	0 0676	49
7	" "	65	150	0 0215	0 0575	18	37	0 0830	0 0585	70
C.										
8	<i>Andropogon sorghum</i> .	50	300		0 0000	0			0 0026	
9	" "	50	300	0 0026	0 1150	18	37	0 1176	0 0650	55

In Experiment 1, given in section A of the table, 50 gm. of the finely comminuted leaves of *Prunus* were distilled at once from 350 cc. of 5 per cent sulfuric acid. In Experiment 2 of the table distillation was made after the comminuted leaves of *Prunus* had macerated in distilled water for 24 hours in a stoppered flask in presence of toluene, enough concentrated sulfuric acid being added just before distillation to make a 5 per cent solution. In other experiments, not tabulated, 50 gm. portions of the comminuted leaves were macerated with 350 cc. of distilled water, or with water and enough toluene to inhibit the growth of microorganisms. After standing 18 to 24 hours enough concentrated sulfuric acid was added to each flask to give a concentration of 5 per cent of sulfuric acid and the mixture was immediately distilled. As appears from section A of the table only a portion of the hydro-

cyanic acid present in the plant was recovered after maceration. In the experiments recorded in section B of the table the same phenomenon in the leaves of *Andropogon sorghum* is further studied. In the case of Experiment 3 the distillation was made at once, as just described for the leaves of *Prunus*. In all of the subsequent experiments the finely comminuted leaves were macerated for varying lengths of time with added potassium cyanide. In Experiment 4 maceration was made at room temperature, while in Experiments 5 to 7 incubation was made at 37°C. For Experiments 8 and 9 of section C of the table 100 gm. of the comminuted leaves of *Andropogon* were heated quickly to the boiling point, and, after cooling, divided into two equal portions. One of these (Experiment 8), after receiving enough sulfuric acid to give a 5 per cent solution, was distilled at once; the other (Experiment 9) was macerated in presence of added potassium cyanide and incubated at 37°C. before distillation from sulfuric acid, as shown in the table. In all cases less hydrocyanic acid was found after maceration, either with or without the addition of cyanide, than could be obtained by distillation of the fresh comminuted leaves. The calculated findings of autogenous hydrocyanic acid are based on the findings given in Experiments 3 and 8.

For purposes of comparison between cyanogenetic and non-cyanogenetic plants, 50 gm. portions of the finely comminuted leaves of *Sambucus canadensis* were distilled at once from 350 cc. of 5 per cent sulfuric acid. No cyanide could be detected in the distillate. Other 50 gm. portions of the leaves of *Sambucus* were allowed to macerate in stoppered flasks for 24 hours in 350 cc. of water in presence of toluene. They were then distilled after adding enough concentrated sulfuric acid to give a 5 per cent solution. No cyanide could be detected in the distillate. To other 50 gm. portions known amounts of a standardized solution of potassium cyanide were added in a total volume of 350 cc. Of these mixtures a number were distilled at once with enough concentrated sulfuric acid to give a 5 per cent solution. All the cyanide added was recovered in the distillate. The other mixtures were not distilled at once, but were allowed to macerate in stoppered flasks in presence of toluene from 6 to 36 hours. All of the cyanide was recovered in these experiments also.

In certain other experiments not recorded in the table 100 gm.



portions of leaves of *Andropogon sorghum* and leaves of *Sambucus canadensis* were macerated with known amounts of added potassium cyanide and 5 gm. of glucose. As controls similar experiments without glucose were performed. After 18 hours of maceration at room temperature, all of the portions were distilled in the manner above described. The amount of hydrocyanic acid recovered was as great from those portions that had been macerated with glucose as from those without it.

It is evident from the experiments reported in section A of the table that the amount of hydrocyanic acid which can be separated by distillation with a 5 per cent sulfuric acid from the leaves of *Prunus virginiana* is greater if they are distilled at once than if they are first macerated. It therefore follows that during maceration the hydrocyanic acid naturally present in the plant is in part either destroyed or converted into a form in which it is not separable by hydrolysis in the usual way. It is evident from experiments recorded in section B of the table that in the case of *Andropogon sorghum* added cyanide can be recovered only in part after maceration. Apparently, the macerating plant tissue exerts the same action upon added cyanide that it exerts upon hydrocyanic acid set free from its glucoside. Results recorded in section C of the table give some indications concerning the nature of the mechanism which is responsible for the disappearance of the cyanide. This experiment shows that in the presence of comminuted sorghum that has been heated, cyanide disappears, at least to as great a degree as in the presence of unheated, comminuted sorghum. It therefore follows that the mechanism is not dependent upon the presence of living protoplasm or of enzymes. Hence it is probable that in the case of sorghum, at any rate, the disappearance of the cyanide is dependent upon some chemical or physical phenomenon. It is probably not due to adsorption by the plant fiber, for, if that were the case, it would have occurred with *Sambucus*. Whether this phenomenon is a reaction with aldehyde or with some other reacting substance, or is some quite different phenomenon remains to be ascertained. It is apparent from the experiments with *Sambucus canadensis* that this phenomenon is not universal in plant leaves. If it is a chemical phenomenon it would be natural to suppose that it might be nitrile formation since most plants contain a variety of aldehydes,

the commonest of which is glucose. That glucose is not concerned in the phenomenon is evident from the experiments above described.

It follows from the results above described, as well as from the statements in the literature, that there are a number of causes for the losses of hydrocyanic acid from plant tissues whereby the accuracy of the determination of hydrocyanic acid in plant tissues is impaired. That these facts must be considered in making such determinations has not hitherto been recognized. Among the causes of these losses are incomplete hydrolysis of the glucoside and disappearance of the hydrocyanic acid during maceration. In making determinations of hydrocyanic acid in plant tissues, therefore, the duration of distillation for the complete removal of the hydrocyanic acid must be determined for each species. Moreover, it is necessary in determining hydrocyanic acid in plants to do so by maceration, by direct distillation, separately, of both the fresh and the macerated plant, and by distillation, after extraction, as suggested by Henry and Auld. In this way the method which yields the greatest quantity of hydrocyanic acid can be determined for each species.

#### SUMMARY.

In this paper it has been shown that the leaves of *Prunus virginiana* must be distilled with acid 4 hours before all of the hydrocyanic acid is liberated, whereas in *Andropogon* and *Panicularia* less than 1 hour is sufficient to liberate all hydrocyanic acid present. It is further shown that in macerating plant tissues that contain hydrocyanic acid a certain amount of the hydrocyanic acid present or of cyanide that may be added is so converted that it is not recoverable by distillation with sulfuric acid. This is not due to the action of enzymes or to the presence of glucose. It is here shown that in determining hydrocyanic acid in plants several methods in corroboration of each other must be used.

## CYANOGENESIS IN PLANTS. STUDIES ON TRIDENS FLAVUS (TALL RED TOP).<sup>1</sup>

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*Tridens flavus* (L.) Hitchcock (1) is a grass sometimes attaining a height of 6 feet. It has been described by different botanists under a variety of names. The most important synonyms are *Sieglingia sesleroides* (Mich.) Scribner, *Triodea cuprea* Jacquin, and *Poa flava* (L.). It is found widely distributed in the United States, occurring from Massachusetts to Kansas and south to Texas. The specimen described by Linnaeus was obtained from Virginia and had a yellow panicle, hence the name *flavus*. The specimen on which our work was done had a purple panicle. This form is the most common in or around the District of Columbia.

In a series of investigations on grasses (2) it was found that *Tridens flavus* yielded hydrocyanic acid. In our present work we used plants collected in August, September, and October, 1914. The hydrocyanic acid was liberated by distilling the plant with dilute sulfuric or tartaric acids and the amounts were determined colorimetrically as Prussian blue (3).

The whole plant, including the roots, collected on Aug. 18 and examined without drying, yielded 0.0075 per cent of hydrocyanic acid. In the plant collected on Sept. 22 we found only a trace of hydrocyanic acid, and none in plants collected in October. That the hydrocyanic acid was not present in the free state in the plant collected in August was shown by the fact that a distillation with steam gave a negative result.

The August plant, after drying in desiccated air at 50°C., retained 0.0066 per cent of hydrocyanic acid, and this quantity still

<sup>1</sup> This paper was presented at the meeting of the Society of Biological Chemists held at St. Louis in December, 1914.

remained after the dried plant had been ground and left exposed to the laboratory atmosphere for 3 months. A sample of the fresh August plant was dried in the laboratory atmosphere for more than a month. When examined at the end of this period it contained only 0.0015 per cent of hydrocyanic acid. Another sample of the same plant, stored for the same length of time at 0°C. gave 0.0013 per cent of hydrocyanic acid. Parts of the fresh August plant, dried in the same manner and for the same length of time, were examined to ascertain the distribution of the hydrocyanic acid in the plant, with the following results:

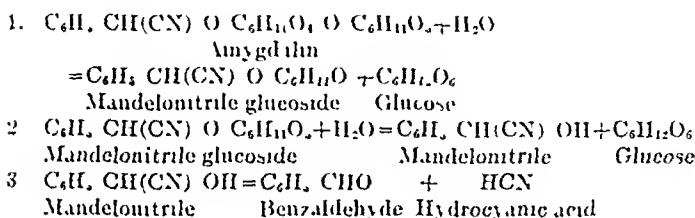
	per cent
Inflorescence tops stripped of flowers . . . . .	0.0037
Stems . . . . .	0.0030
Green leaves . . . . .	0.0017
Dead yellow leaves.....	0.0000
Root. ....	Trace.

The flowers selected for this experiment became mouldy, but after drying still gave about 0.0002 per cent of hydrocyanic acid. An analysis of flowers from the August plant, which had been stored at 0°C., was made and 0.0025 per cent of hydrocyanic acid was found. The very small amount of this acid found in the September plant was located chiefly in the unripe seeds. Fresh ripe seeds from the October plant gave a negative test.

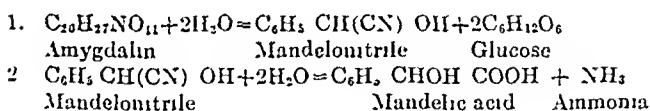
We found that when *Tridens* was macerated for some time in water before distilling with acid, a considerable loss of hydrocyanic acid ensued. This result agrees with recent observations made by Alsberg and Black (4) on other plants and may have an important bearing on the results obtained by other workers in this line. For instance, in the investigations of Treub (5) on a large number of plants, the quantity of hydrocyanic acid obtained was increased by macerating the plant before distilling. Apparently in a number of cases where such an increase was observed after maceration, the material was distilled without the addition of a strong acid. If the macerated or unmacerated plant had been distilled together with a strong acid, different results might have been obtained, depending, of course, on the character of the cyanogenetic compound present.

Many cases are well known where hydrocyanic acid is liberated from plant material during maceration in water. The liberation

of the hydrocyanic acid in such cases is due to an enzymic reaction. For example, when bitter almonds are macerated with water, the enzyme emulsin (6), which is present together with the amygdalin, decomposes this cyanogenetic compound mainly in such a manner that the hydrocyanic acid is set free. The enzyme reaction proceeds as follows:



On the other hand, if the bitter almonds are distilled under usual conditions with acids without previous maceration the main reaction is different, resulting in the formation of mandelonitrile and glucose, only a small quantity of hydrocyanic acid being obtained.\* The mandelonitrile is further hydrolyzed by acids to mandelic acid and ammonia. These reactions may be shown as follows:



It has also been observed that when certain plants are macerated in dilute solutions of potassium cyanide (4) a large quantity of the potassium cyanide cannot be recovered as hydrocyanic acid by subsequent distillation with a stronger acid. *Tridens* belongs to this class of plants. When known weights of potassium cyanide were added to a mixture of the plant and water in tightly stoppered flasks which were kept at temperatures varying from 0-30°C., a loss of potassium cyanide occurred in every case and this loss increased with the period of maceration. When sodium hydroxide was added to the above mixture the loss of cyanide was

\* Experiments on the distillation of amygdalin with acids under varying conditions have been undertaken and will be reported in another paper

much greater. On the other hand, when tartaric acid was added to the mixture of plant, water, and potassium cyanide, there was no loss of cyanide on distilling after maceration. The loss of cyanide might be due to a chemical reaction between some of the compounds contained in the plant and the potassium cyanide. It is well known that cyanides react with a large number of organic compounds, such as aldehydes and ketones.<sup>3</sup>

The loss of cyanide during maceration might also be ascribed to an enzymic action, such as the action of a plant oxidase that could change the potassium cyanide to potassium cyanate.

In this connection it might be well also to consider the action of bacteria which could grow in the maceration mixture.

Adsorption of the potassium cyanide by the plant during the period of maceration might also account for this loss, since it has been found that vegetable fibers adsorb salts (7).

To ascertain if volatile aldehydes or ketones were present, which would react with the cyanide, we made an infusion of the plant with water and distilled over more than one-half of the liquid. Potassium cyanide was allowed to stand in this distillate, and on acidifying and distilling there was no loss of cyanide. A portion of the distillate was made alkaline with sodium hydroxide, potassium cyanide was added, and after standing a day all the cyanide was recovered. Hence the loss of potassium cyanide was not due to volatile aldehydes or ketones.

The plant mentioned above, which had been heated more than 1 hour during the distillation, still had the power of acting on potassium cyanide, the loss being over 20 per cent on macerating for 27 hours at 15°C. As the distillation removed only volatile products, it is still possible that the loss of potassium cyanide was due to a chemical reaction.

Attempts were also made to prevent enzyme action by boiling the plant in alcohol and by macerating with dilute alcohol. On subsequent distillation, either with or without the addition of acids, only a part or none of the available hydrocyanic acid was obtained.

<sup>3</sup> Dezanì has shown that hydrocyanic acid is transformed to ammonia by vegetable juices (*Atti II Cong. naz. chim. appl.*, Turin, 1911). Our attention was called to this paper after we had been forced to discontinue our experiments on account of lack of material.

To exclude definitely the action of enzymes the October plant was heated in dry air up to 130°C. for 4 hours. On maceration with potassium cyanide at 15°C. for 43 hours, the loss of cyanide was about 60 per cent. The August plant, heated in the same manner, although it lost three-fourths of its available hydrocyanic acid, still acted on potassium cyanide under similar conditions, also causing a loss of about 60 per cent.

To exclude the action of bacteria and enzymes and the adsorption of potassium cyanide by plant fibers, an aqueous extract from the October plant was filtered and sterilized. Potassium cyanide was added to this liquid and a loss of 40 per cent of cyanide was observed after the mixture had stood at 15°C. for 45 hours. These results indicate that the loss of potassium cyanide is due to a chemical reaction.

That an enzyme is present in *Tridens* was shown by the action of the October plant on a solution of amygdalin. In 20 hours, 34 per cent of the amygdalin was hydrolyzed. The action of emulsin on *Tridens flavus* was tried and the same quantity of hydrocyanic acid was found as in a control experiment without emulsin.

When more material is available we intend to continue our studies on this plant and attempt to isolate the cyanogenetic compound and the enzyme.

#### EXPERIMENTAL PART.

The general method employed to obtain the hydrocyanic acid from the plant was as follows: The plant was placed in a flask of convenient size. The material was then covered with a measured volume of water, and sulfuric, or usually solid tartaric, acid was added to make a 5 to 10 per cent solution. The flask was immediately attached to a condenser, to the end of which a delivery tube was fitted. This tube was dipped into a dilute solution of sodium hydroxide. The mixture was boiled until the distillate no longer gave a test for hydrocyanic acid. In the case of *Tridens* all the available hydrocyanic acid was obtained before one-half of the water had distilled over. The volume of the distillate was ascertained, and an aliquot part was analyzed by converting the cyanide to Prussian blue and estimating this colorimetrically (3).

*Determination of Hydrocyanic Acid in Tridens Collected on Aug. 18.*

250 gm. of the whole fresh plant, including the root, were distilled without previous maceration, as described above. The yield of hydrocyanic acid was 0.0075 per cent.

A large sample of the above mentioned plant was dried at 50°C. in a stream of desiccated air. This material was then ground and left exposed to the laboratory atmosphere. Portions were analyzed at different intervals up to Nov. 9. All these analyses gave 0.0066 per cent of hydrocyanic acid.

*Effect of Maceration on the Yield of Hydrocyanic Acid.*

To determine the influence of maceration, experiments were made with the ground August plant dried as mentioned above. This contained 0.0066 per cent of hydrocyanic acid. The results given in Table I indicate that maceration diminished the quantity of available hydrocyanic acid.

TABLE I.

*Effect of Maceration on the Yield of Hydrocyanic Acid.*

Weight of plant.	Water added.	Time of maceration.	Temperature of maceration.	HCN in plant as KCN.	HCN recovered as KCN.	Loss of HCN.
gm.	cc.	hrs.	°C.	gm.	gm.	per cent
50	600	6	15	0.008	0.0057	29
50	600	18	15	0.008	0.0047	41
50	600	18	Room.	0.008	0.0040	50
100	1,000	20	"	0.016	0.0091	43
50	600	23	15	0.008	0.005	38
50	600	42	15	0.008	0.0052	35
50	600	65	15	0.008	0.0034	58
25	300	2	34	0.004	0.0036	10
25*	300	2	34	0.004	0.0036	10
25	300	24	34	0.004	0.0000	100
25*	300	24	34	0.004	0.0000	100

\* 0.5 gm. of emulsin added.

*Effect of Distilling with and without the Addition of Acid under Varying Conditions.*

Experiments were made to determine the effect of distilling the macerated or unmacerated plant without the addition of acid. It was found that in every case distillation without the addition



of acid resulted in a partial or complete loss of the available hydrocyanic acid. Similar experiments were made in which dilute alcohol was used instead of water. The losses here were more pronounced. The results of these experiments are shown in Table II.

TABLE II

*Effect of Distilling with and without the Addition of Acid under Varying Conditions.*

Weight of plant	Water added	Time of maceration	Temperature of maceration	HCN in plant as KCN	HCN recovered as KCN.	Loss of HCN	Manner of distilling
gm	cc	hrs	°C	gm	gm	per cent	
250	1,000	0	—	0 015	0 015	—	With acid.
250	1,000	0	—	0 015	None.	100	Steam without acid.
25	300	0	—	0 004	Trace	Near-ly 100	Without acid
25	Hot water. 300	Boiled in water 30 min	—	0 001	0 00075	81	" "
25	300	20	15	0 001	0 0036	10	With acid
25	300	20	15	0 004	0 0030	25	Without acid
25	300	26	15	0 004	0 003	25	" "
25	300	18	15	0 004	0 0028	30	" "
25	500	0	—	0 004	None	100	" "
25	50 per cent alcohol 100	Boiled in 200 cc of 95 per cent alcohol 30 min	—	0 004	"	100	" "
25	500 50 per cent alcohol	Boiled in the 50 per cent alcohol 15 min, macerated 30 hrs	Room	0 004	0 0023	42	With acid
25	500 50 per cent alcohol.	40	"	0 004	0 001	75	Without acid

TABLE VI.

*Nitrous Oxide and Ether Anesthesia.  
Laparotomies.*

Duration.	No. of cases.	A.	B.	C.	Increase of B over A.
<i>min.</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
20-30	4	0.102	0.140	0.096	37
30-40	7	0.090	0.118	0.083	31
40-60	5	0.094	0.120	0.093	27
60-90	9	0.097	0.161	0.134	66
Over 90	2	0.089	0.143	—	61
All cases.	27	0.096	0.138	0.109	44

TABLE VII.

*Nitrous Oxide and Ether Anesthesia.  
Extraperitoneal Operations.*

Duration.	No. of cases.	A.	B.	C.	Increase of B over A.
<i>Gynecological cases.</i>					
<i>min.</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
10-20	1	0.080	0.104	0.084	30
20-30	4	0.098	0.123	0.083	26
30-40	2	0.094	0.131	—	40
40-60	5	0.105	0.141	0.112	34
60-90	2	0.085	0.112	0.103	32
All cases	14	0.097	0.128	0.100	32

*Renal cases.*

60-90	3	0.089	0.154	0.102	73
110	1	0.087	0.204	0.096	134
All cases	4	0.088	0.166	0.100	89

*Neurological cases.*

60-90	2	0.091	0.142	0.106	56
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*Miscellaneous cases.*

20-30	4	0.084	0.112	0.109	33
30-40	3	0.102	0.123	0.102	21
40-60	2	0.105	0.121	0.101	15
60-90	3	0.089	0.156	0.101	76
All cases. . . . .	12	0.093	0.127	0.104	37

rotomies; (2) extraperitoneal operations. This general grouping is made because of the reputed effect of intraperitoneal operations on the blood sugar content. In the accompanying tables the results are grouped according to the above scheme, specifying, however, the location of each group of operations and the duration of the anesthesia. Column A represents the average sugar content of the blood examined just before anesthesia; Column B represents the average sugar content directly after the operation; and Column C represents the sugar content 24 hours after operation.

In fifty-seven of the sixty cases examined there was a well marked increase in the blood sugar content after anesthesia and operation. In one case (No. 9, a plastic lasting 70 minutes) there was a rise of only 0.001 per cent within the limit of error. In another case (No. 39, Table III, a urethroplasty lasting 50 minutes) there was a decrease of 0.004 per cent, in a third a decrease of 0.006 per cent.

The average results in all kinds of cases, regardless of duration, showed a decided increase after operation, and the persistence of a moderate increase 24 hours later. Reference to Tables VI, VII, VIII, and IX will give the figures for the different groups of cases studied.

TABLE VIII.

*Nitrous Oxide and Ether Anesthesia.  
Summary, According to Nature of Operation.*

Nature of operation	No of cases	A.	B	C.	In-crease of B over A
		per cent	per cent	per cent	per cent
Intrap <del>eritoneal</del> <sup>peritoneal</sup>	27	0 096	0 138	0 109	44
Gynec <del>eritoneal</del> <sup>peritoneal</sup>	14	0 097	0 128	0 100	32
Rena <del>l</del> <sup>l</sup>	4	0 088	0 166	0 100	89
Neur <del>al</del> <sup>al</sup>	2	0 091	0 142	0 106	56
Misc <del>aneous</del> <sup>aneous</sup> including	12	0 093	0 127	0 104	37
Heit <del>as</del> <sup>as</sup>	4	0 099	0 138	0 106	40
Th <del>of</del> <sup>of</sup> oids (not exophthalmic)	2	0 093	0 133	0 110	43

In cases (Table IX) which were under anesthesia for less than 1 hour the average percentage increase of B over A varied from 15 to 37 per cent. The increase of only 15 per cent was seen in two

TABLE IX.

*Nitrous Oxide and Ether Anesthesia.**Laparotomies and Non-laparotomies, Grouped According to Duration of Anesthesia.*

	No. of cases	A.	B.	C	Increase of B over A
		per cent	per cent	per cent	per cent
Duration 20-30 min					
Laparotomies	4	0.102	0.140	0.096	37
Non-laparotomies	8	0.091	0.118	0.096	30
Duration 30-40 min					
Laparotomies	7	0.090	0.118	0.083	31
Non-laparotomies	5	0.098	0.127	0.102	30
Duration 40-60 min.					
Laparotomies	5	0.094	0.120	0.093	27
Non-laparotomies	7	0.105	0.135	0.109	30
Duration 60-90 min.					
Laparotomies	9	0.097	0.161	0.134	66
Non-laparotomies	10	0.089	0.144	0.103	62
Of these,					
Renal cases	5	0.090	0.149	0.103	65
Others	5	0.087	0.138	0.102	59

miscellaneous extraperitoneal procedures lasting 40 to 60 minutes. The increase of 37 per cent was seen in a group of four laparotomies lasting 20 to 30 minutes.

In cases (Table IX) which were under anesthesia for more than 1 hour the average percentage increase varied from 32 to 89 per cent. The increase of 32 per cent was seen in two gynecological cases, plastic operations lasting 60 to 90 minutes. The increase of 89 per cent was seen in a group of four nephrectomies lasting from 60 to 110 minutes.

The greatest percentage increase of B over A was seen in this group of nephrectomies (Table VIII). In Case 1 (nephrectomy for hydronephrosis, lasting 110 minutes) the readings were A, 0.087, B, 0.204, C, 0.096 per cent, a rise of 134 per cent of B over A, the largest increase in the series of cases examined.

From a study of Fig. 1 we conclude that: (a) There was no difference between the effect of intraperitoneal operations and extraperitoneal operations upon the sugar content of the blood. (b) The anesthesia played the important rôle in the production

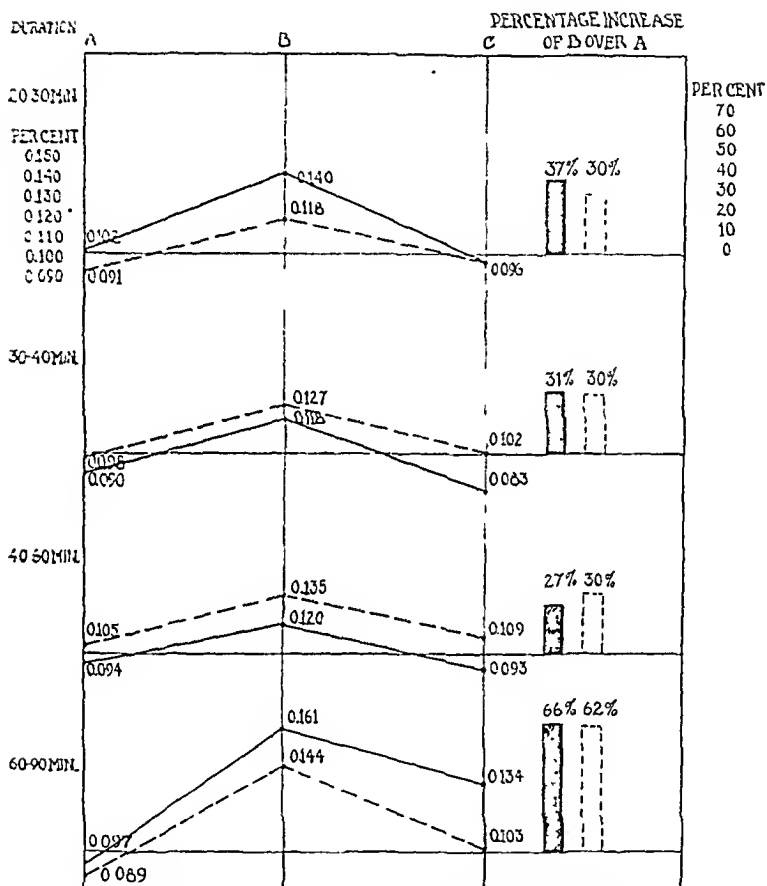


FIG. 1. Chart showing the course of the glycemia (Table IX). A, before; B, at end of anesthesia; C, 24 hours later. Vertical lines, percentage increase of sugar after anesthesia of various duration. Continuous lines, laparotomies. Broken lines, extraperitoneal operations.

of the hyperglycemia observed. (c) The more marked increases in blood sugar content appeared when the anesthesia lasted 1 hour or more. (Bang stated that deep narcosis produced hyperglycemia of its own accord.) (d) The operation of nephrectomy had an especially marked effect in the blood sugar content and the operation of itself was responsible for a good part of the hyperglycemia, over and above the effect of the prolonged anesthesia.

*Loss of Blood.*—As stated previously, loss of blood is known to produce a hyperglycemia. It is difficult to specify the degree of influence which this factor played in our cases. From an analysis of the clinical data it may be stated that excessive bleeding was not encountered. Certain cases, such as No. 42 (Wertheim operation), in which a moderate loss of blood was incidental, showed a somewhat greater rise than other cases, but not much beyond the range of the average.

*Post-operative Glycosuria.*—This was found in only one of fifty cases in which the post-operative urine was examined for sugar with Fehling's reagent. This was a plastic operation lasting 55 minutes (Case 40). The readings for this case were A, 0.118, B, 0.232, and C, 0.200 per cent.

The urine passed 8 hours after operation gave a well marked reduction. A specimen voided 24 hours later gave no reduction. The persistence of a well marked hyperglycemia 24 hours after operation was unusual.

In view of the fact that anesthesia produces a well marked hyperglycemia in almost every case, we would expect glycosuria to result more often. That we do not find it may be due to one of two causes: (1) The percentage increase of blood sugar may be relative and not absolute, just as in the case of loss of blood. (2) The kidneys may be rendered impermeable to sugar by the anesthetic. Further work upon this phase of the subject is in progress.

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positive diagnosis of cancer and pregnancy. Echols<sup>20</sup> states that the dialysis method is of value when negative, showing the absence of pregnancy, but that a positive result may be due to errors in technique. Carlson,<sup>21</sup> in answer to the last two statements, says that the Abderhalden test is not qualitative but quantitative, and that one could probably get a positive test in 100 per cent of the cases if the time for digestion were extended, as polyvalent ferments are present always in the blood. This latter position is supported by many investigators. Flatow<sup>22</sup> finds that the splitting of placenta occurs not only in the pregnant serum, but also that the proteolytic ferments of the normal serum break up the proteins of the placenta unspecifically and quantitatively. He<sup>23</sup> also objects to Abderhalden's results on the basis of his using "cooked" placenta, saying that "if specific ferments were present they would not show with cooked organs." In his later work<sup>24</sup> with casein, he finds that casein is digested by the normal serum and more intensely with the pregnant serum. Michaelis and Lagermark<sup>25</sup> were not able to demonstrate a specific ferment for placenta, but obtained positive results even with men and old women in certain pathologic conditions. The results of many investigators indicate the presence of proteolytic ferments which evidently increase in quantity under certain conditions.

Herzfeld<sup>26</sup> found the percentage of amino-acid greater in pregnant than in normal serum, indicating that the pregnant serum had increased fermentative powers. Schlumpert and Issel<sup>27</sup> report increased ferments in animals during pregnancy, but they were not specific for the animals' own placenta. According to the recent investigations of Jobling, Eggstein, and Petersen,<sup>28</sup> placental tissue is not digested but actually resists, in an increased degree, enzyme action. Kolmer and Williams<sup>29</sup> are of the opinion that during pregnancy there is an increase of the general proteolytic ferment of the serum; and recently they<sup>30</sup> state that there are two sets of ferments in pregnant serum; (1) normal non-specific ferment, and (2) specific; the former released through the adsorption of the antiferment by non-specific substances, the second released only through specific protein antigen.

<sup>20</sup> Echols, C. M., *J. Am. Med. Assn.*, 1914, lxiii, 370.

<sup>21</sup> Carlson, A. J., *J. Am. Med. Assn.*, 1914, lxiii, 1176.

<sup>22</sup> Flatow, L., *Munch. med. Woch.*, 1914, lxi, 468.

<sup>23</sup> Flatow, *Munch. med. Woch.*, 1914, lxi, 1168.

<sup>24</sup> Flatow, *Munch. med. Woch.*, 1914, lxi, 1500.

<sup>25</sup> Michaelis, L., and Lagermark, L. v., *Deutsch. med. Woch.*, 1914, xl, 316.

<sup>26</sup> Herzfeld, E., *Biochem. Z.*, 1914, lix, 249.

<sup>27</sup> Schlumpert, H., and Issel, E., *Munch. med. Woch.*, 1913, lx, 1758.

<sup>28</sup> Jobling, J. W., Eggstein, A. A., and Petersen, W., *J. Exp. Med.*, 1915, xxi, 239.

<sup>29</sup> Kolmer, J. A., and Williams, P. F., *Am. J. Obst.*, 1915, lxxi, 899.

<sup>30</sup> Kolmer and Williams, *Am. J. Obst.*, 1915, lxxii, 1.



The immunologists have also offered explanations for this increased activity during pregnancy and after injection of protein, as an immunity reaction. Vaughan<sup>31</sup> states that he procures in guinea pigs, by immunization, a serum which will digest egg protein. He explains immunity as development of a serum which will digest foreign bodies and render them harmless. Jobling and Petersen<sup>32</sup> explain this digestion in blood as due to non-specific proteolytic ferments or proteases normally present in blood and held in check by antiferments, which they believe to be unsaturated fatty acids. Bronfenbrenner<sup>33</sup> explains the reaction by saying that the results obtained by Abderhalden are due to the presence in this serum of specific substances that are not fermentative in nature, and that the substratum is not digested, but that autolysis takes place and serum proteins are liberated, thus giving rise to protein digestion products in the blood. On the other hand, we have the evidence of Frank<sup>34</sup> that no experimental specific immune reaction to placenta can be demonstrated. Lake<sup>35</sup> also regards the possibility of producing an immune serum of therapeutic value in chorion epithelioma, by use of human placenta, as extremely slight. Abderhalden<sup>36</sup> regards the diagnosis of pregnancy and carcinoma as dependent on the presence of ferments produced by the injection of the protein or tissue in question. De Waele,<sup>37</sup> however, did not succeed in preparing a serum that would give digestion with specific tissues, but he did find that the addition of even an inorganic substance will cause the liberation of dialyzable, ninhydrin-reacting substances. DeWaele,<sup>38</sup> and Heilner and Petri<sup>39</sup> show that ferments appear very quickly after parenteral injection of the protein, in intervals hardly sufficient for the elaboration of new and specific ferments; they support the theory that the ferments are preformed and that the substratum serves to activate rather than to bring about the production of new ferments.

The most recent work done on proteoclastic ferments tends to favor the opinion that the production of ferments during pregnancy and after the injection of protein is a quantitative and not a qualitative reaction. Sloan<sup>40</sup> observed that there may be an increase of proteolytic activity un-

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<sup>31</sup> Vaughan, V. C., *J. Am. Med. Assn.*, 1914, lxiii, 365.

<sup>32</sup> Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xix, 459; *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 356.

<sup>33</sup> Bronfenbrenner, J., Sehlesinger, M. J., and Mitchell, W. T., *J. Am. Med. Assn.*, 1915, lxxv, 1268.

<sup>34</sup> Frank, R. T., *J. Exp. Med.*, 1907, ix, 263.

<sup>35</sup> Lake, G. C., *J. Infect. Dis.*, 1914, xiv, 385.

<sup>36</sup> Abderhalden, *Fermentforschung*, 1914, i, 20.

<sup>37</sup> De Waele, H., *Z. Immunitätsforsch., Orig.*, 1914, xxii, 170.

<sup>38</sup> De Waele, *Z. Immunitätsforsch., Orig.*, 1909-10, iv, 148.

<sup>39</sup> Heilner, E., and Petri, T., *Munch. med. Woch.*, 1913, lx, 1530.

<sup>40</sup> Sloan, LeR. H., *Am. J. Physiol.*, 1915, xxxix, 9.

der the above conditions. Van Slyke, Vinograd-Villehur, and Losee<sup>41</sup> found nearly or quite as much power to digest placenta in normal as in pregnant sera. Malone<sup>42</sup> reported that he has found ferments, even in the urine, which would digest placenta. The clinical literature on the subject, supporting the efficiency of the test and also denying its usefulness, would fill volumes and has been omitted here, because we are concerned with the reaction from the chemical rather than from the clinical viewpoint. A full list of references may be found in Abderhalden's *Abwehrfermente*.

#### EXPERIMENTAL.

As stated in the introduction, the object of this investigation was to determine whether proteoclastic ferments or activity develop in the blood in response to the injection of a foreign protein and cause digestion when allowed to act on the protein injected, using a method which would not be open to the criticism of the dialysis method or of the ninhydrin reaction. The method followed in each case was practically the same. White rabbits were injected with an amount of protein varying from 100 to 800 mg. in solution or suspension, depending on its solubility in Ringer's solution. The Ringer's solution was boiled and cooled to about 40°C. The protein was placed in this and after the hair had been cut from the rabbit and the skin disinfected with iodine, this solution was injected parenterally. Each rabbit received three successive injections, 1 week apart. The day following the last injection the rabbit was etherized and bled to death from the carotid. The blood was caught in a beaker and was stirred continuously to defibrinate it. The control animals were bled in the same way. The defibrinated blood was centrifuged, the serum decanted off, and portions were used to obtain the figures for the non-protein nitrogen according to the method of Folin,<sup>43</sup> except that we used, for some of the later tests, absolute ethyl alcohol instead of methyl alcohol.

The rest of the serum was divided into 5 or 10 cc. portions ac-

<sup>41</sup> Van Slyke, D. D., and Vinograd, M., *Proc. Soc. Exp. Biol. and Med.*, 1914, xi, 154. Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 377.

<sup>42</sup> Malone, R. H., *Proc. Soc. Exp. Biol. and Med.*, 1915, xii, 126.

<sup>43</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

cording to the amount obtained, and these were diluted up to 50 cc. with Ringer's solution, made alkaline with 2 cc. 0.1 N NaOH and placed in a flask with 1 gm. of the particular protein, and incubated at 37.5°C. The first three were incubated for 24 hours, the next two for 48 hours, and the last two for 42 hours. After incubation the contents of the flask were mixed with ten volumes of alcohol, and allowed to stand for 24 hours. The filtrate was treated with a saturated alcoholic solution of  $\text{ZnCl}_2$ , allowed to stand 24 hours, and a Kjeldahl run on the filtrate. Bearing in mind the fact that Greenwald<sup>44</sup> could not recover all the amino-acid from an alcoholic filtrate, we did some check analyses with 2.5 per cent trichloroacetic acid, as recommended by him, but since the results checked so closely with the alcohol figures, we continued with the alcohol method.

The results are summarized in the tables.

TABLE I.

Experiment.	Animal.	Injection.			Non-protein N in 100 cc.	
		1.	2.	3.	Before digestion.	After digestion.
		mg.	mg.	mg.	mg.	mg.
Protamine	Experimental.	100	100	100	.....	0.039
	" " " "	100	100	100	.....	0.042
	Normal . . . . .	...	...	...	.....	0.048
Casein	Experimental....	500	500	500	0.040	0.041
	Normal . . . . .	...	...	...	0.021	0.037
Bence-Jones	Experimental. .	250	125	250	0.034	0.059
	Normal.. . . .	...	...	...	0.037	0.060
Phaseolin	Experimental	800	500	500	0.036	0.039
	Normal... . .	...	...	...	0.039	0.035
Edestin.	Experimental	500	450	400	0.039	0.045
	Normal .. . . .	...	...	...	0.036	0.041
Gliadin	Experimental.	350	450	400	0.035	0.039
	Normal . . . . .	...	...	...	0.100	0.100
Soy bean globulin	Experimental.	250	250	250	0.064	0.062
	Normal . . . . .	...	...	...	0.036	0.047
Milk albumin	Experimental .	250	250	250	0.037	0.052
	Normal . . . . .	...	...	...	0.036	0.045

<sup>44</sup> Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

TABLE II.

Digestion with	Increase of non-protein N.	
	Injected animals	Normal animals
	mg.	mg.
Protamine . . . . .	..	..
Casein.....	1	16
Bence-Jones... ..	25	23
Phaseolin.....	3	- 1
Edestin.... .	6	5
Gliadin.....	4	0
Soy bean globulin..	-2	11
Milk albumin.. .	15	12

# DISCUSSION.

The results show plainly in all cases that under the conditions of the experiment there is practically no digestion with the blood of the injected animal in excess of that which takes place with the serum of the normal control animal. The greatest amount of digestion takes place in the case of the Bence-Jones protein and milk albumin, which would naturally be expected, as these two proteins are more nearly related to mammalian proteins. With casein, the blood of the injected animal on the addition of casein gave a precipitin reaction, so that this might account for the greater increase of nitrogen in the normal animal; the precipitin reaction might preclude the possibility of a proteolytic action taking place. The results would also lead one to suppose that the blood of the rabbit possesses to a slight degree proteolytic activity, but that this activity toward a particular protein is not increased by injecting the animal with that protein. The criticism might be offered that these proteins are too foreign; but if the reaction must be discriminated on the basis of the protein being just foreign enough, it would seem that the possibility of usefulness becomes remote.

The very premise that ferments (free or restrained) exist preformed in the blood is not strongly borne out in these data. The ferments are best regarded as endocellular entities; and under certain conditions, not well understood, the cellular activity may be so stimulated that an excess of ferment is produced and overflows

into the blood stream (just as frequently trypsin occurs in the urine), but exists there not primarily for the purpose of digestion directly in the circulating blood. The fact that such varying results on the presence or activity of ferments in the blood have been obtained might be due to the fact that some of the investigators have accidentally reproduced the conditions under which the cells are stimulated to greater activity and the excess ferments then appeared in the blood. Our results would lead us to believe that pure proteins at least need not call forth such increased activity. Even though there be in pregnancy a specific serum reaction, it is probably not one due to placental digestion in the blood.

It is not demonstrated that the normal hydrolysis of the protein of the body occurs in the circulating blood; indeed, it is much more likely that this occurs largely within the cells of the tissues. It is easily possible to imagine introduced foreign protein being taken into the body cells and hydrolyzed there, without any reaction appearing in the circulating plasma. It may be shown that foreign protein introduced into the venous blood may be taken up by the tissues.<sup>45</sup> In like manner, it might be imagined that the placental cells entering the maternal circulation would be held and hydrolyzed within the tissues, and leave no trace of any enzymic or other activity in the circulating plasma. In theory, therefore, one must separate the question of hydrolysis of foreign proteins from the question of the reaction of the maternal body to placental cells. It is entirely possible that there is a specific reaction of the host to placental cells or to neoplasms, without this being a hydrolysis of their proteins in the circulating blood dependent on the presence there of enzymic activity.

#### SUMMARY.

Protamine, phaseolin, and gliadin are not digested to any degree by either normal serum or that of an animal injected with these substances.

Casein and soy bean globulin are digested to a greater extent by the normal serum than by that of the injected animal.

<sup>45</sup> Unpublished data.

Edestin and milk albumin are digested to the same degree by the normal and experimental serum.

Bence-Jones protein is digested to a marked degree by both sera and equally well in each case.

I take this opportunity to thank Professor Alonzo E. Taylor, at whose suggestion this work was undertaken and under whose direction it was carried out, for his help, both in the experimental work and in reading and revising the manuscript. I also thank Professor Mendel and Dr. Osborne for the proteins<sup>46</sup> supplied by them, Dr. Isaac F. Harris,<sup>47</sup> for those supplied by him, and Drs. Taylor and Miller for the Bence-Jones protein.

<sup>46</sup> Casein, edestin, gliadin, phaseolin.

<sup>47</sup> Soy bean globulin, milk albumin.

*Sugar Data.*

Blood sugar content at		Urine	Blood sugar content at		Urine
10 a m	1 p m		10 a m	1 p m	
200 gm glucose			200 gm glucose		
<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
0 09	0 09	Slightly +	*0 08	0 10	Slightly + +
0 10	0 10		0 10	0 09	
0 12	0 08		0 11	—	
0 11	0 11		300 gm. glucose		
0 12	0 12		*0 10	0 16	
0 11	0 15		0 14	0 16	
0 12	0 12	0 11	0 13	+	
0 08	0 07	*0 07	0 14		
0 09	0 08	0 13	0 14		
*0 08	0 13	0 11	0 13		
*0 09	0 12	0 10	0 10	+	
0 08	0 09	0 07	0 09		
0 09	0 10	*0 10	0 14		
0 07	0 09	+	400 gm glucose		
0 08	0 09	+	0 14	0 12	—
*0 07	0 10		0 07	0 07	—
0 10	0 09		*0 10	0 13	+
0 10	0 07		0 07	0 09	Slightly +
0 08	0 10	+	0 10	0 09	—
*0 06	0 11		0 09	0 09	—
0 10	0 07				
*0 12	0 15				

\* The asterisks indicate cases where there was a rise in blood sugar greater than might be due to the experimental error in the method

From the figures it is clear that nearly all the subjects tolerated the ingestion of 200 gm. without exhibition of glucosuria. Of nine subjects who ingested 300 gm. only three displayed glucosuria. Of the six who ingested 400 gm. only two had glucosuria. The figures for the blood sugars run within the limits to be observed with the use of the Bang method. In some instances the figure is at the very upper limit of the normal, but these figures were by no means observed following the largest ingestions. The figures for blood sugar are of relative value only, because of the time fixed for the estimations, 3 hours after ingestion. It was to be expected that this arbitrary time was too early for the top of

the wave in some subjects, too late in others. The general conclusion may be drawn, however, that such ingestions do not markedly influence the sugar content of the blood.

Two points in the technique required checking. Were results of tests done 2½ to 3 hours after a light breakfast as described, assumed to hold for the empty stomach? To determine this point, 400 gm. of glucose were given four times, without blood examinations, in each instance with negative results. Did the presence of lemon juice, sometimes given to flavor the large mass of syrup, modify the result? 400 gm. were given four times, without lemon juice, on the empty stomach, with negative results. Are 400 gm. the limit of ingestion? In five instances, without blood examinations, 500 gm. were given, with the production of glucosuria in but one. We regard 500 gm. as the physical limit of ingestion, except in one who has trained to the test; it is very large in bulk, inclines to nauseate, and apparently the excess is not rapidly absorbed, so that the test probably means no more than does the administration of 400 gm., which is usually tolerated.

Polyuria occurred rarely, and there was no relationship between the polyuria and glucosuria. Intestinal disturbances were not observed. An attempt was made to repeat the series with levulose, beginning at 300 gm. on account of scarcity of the pure material. But we found that, as a rule, our healthy students could not ingest 300 gm. of levulose without intestinal disturbances. 400 gm. administered to three men caused profuse diarrhea with copious watery stools, without sugar in the sparsely secreted urine. Whether this result is inherent in such amounts of levulose or whether due to some impurity in the supposedly pure preparation we used, could not be determined.

Apparently there is in the majority of healthy adult males no limit of assimilation of glucose; glucosuria does not occur following the largest possible ingestions of pure glucose. These results confirm the findings of Woodyatt, Sansum, and Wilder.<sup>1</sup>

<sup>1</sup> Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assn.*, 1915, lxx, 2067.





# THE QUANTITATIVE DETERMINATION OF THE TOTAL PROTEIN AND NON-PROTEIN SUBSTANCES OF MUSCLE.

## IMPROVED TECHNIQUE.

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A method for the quantitative determination of the protein and non-protein muscle substances was described in a previous communication.<sup>1</sup> The principle involved was coagulation of the protein substances with aid of acetic acid and sodium chloride, followed by separation from the non-protein material through extracting with water, alcohol, and ether. It has, however, been ascertained as a result of added experience that the technique can be considerably improved. Particularly when the muscle was not fresh it was observed that a sample could rarely be met with in which the protein failed to be rendered entirely insoluble by acetic acid and saline, even when the coagulation was carried out with the greatest pains. On investigation, the loss of protein arising from this cause usually proved inconsiderable, but in the three highest instances it was found to lie between 1.0 and 3.5 per cent. The subsequent filtering and washing was tediously delayed under these circumstances.

Owing to the need of a reliable method for the determination of the meat proteins, efforts were made to develop a more satisfactory procedure. No departure was made from the principle of direct determination of the protein substance inasmuch as a complete separation of this material from the non-proteins is also essential for an accurate indirect determination of the protein by means of nitrogen analyses. Consequently, in seeking for a more

<sup>1</sup> Janney, N. W., and Csonka, F. A., *J. Biol. Chem.*, 1915, **xii**, 195.

TABLE II.

*Control Muscle Analyses.*

Species	Muscle for analysis	Protein found		Protein recovered from extracts	Error due to	
					Protein lost in extracts	Ash in protein found
	gm	gm.	per cent	mg	per cent	per cent
Rabbit	10 0053	1 6250	16 2			+0 055
Cat I*	9 9945	1 7548	17 6	38 7	-0 39	+0 09
" II.	9 9860	1 7820	17 8	33 8	-0 34	
Dog I	10 0030	1 729	17 3	29 4	-0 29	+0 08
" II	9 9995	1 722	17 2	29 8	-0 30	
Fish (halibut)	9 9935	1 6705	16.7	16.4	-0 16	+0 05
Ox	10 0000	1 7230	17 2	24 7	-0 25	
"	10 0000	1 4060	14 1	11 2	-0 05	+0 01
" I	10 0109	1 6995	17 0	6 3	-0 06	+0 07
" II	10 0143	1 7008	17 0	8 0	-0 08	
Man I**	10 0000	1 2010	12 0	1 1	-0 01	+0 04
" II ..	10 0000	1 2035	12 0	4 0	-0 04	
" . . .	9 9930	1 7417	17 4	2 4	-0 02	+0 05
" **	10 0095	1 4595	14 6	7 5	-0 07	+0 02
" I .	10 0175	1 6971	16 9	10 2	-0 10	+0 07
" II .	9 9815	1 6548	16.6	18 3	-0 20	

\*Duplicate analyses thus designated.

\*\*From emaciated subject.

The extent to which the percentage values of protein found were influenced by this source of error has been calculated from the results of ash determinations made from this material (Table II). This error is on the positive side and thus serves to counterbalance,

latter are not precipitated by trichloroacetic acid, the difference in the results obtained by these protein precipitants may be ascribed to this cause. Evidently albumoses and peptones are present in very small quantities in fresh meat.

at least partially, the loss of material of protein nature in the extracts described above.

The protein preparations obtained by aid of the new analytical procedure were, as hitherto, also tested for the presence of carbohydrates. Carbohydrates could only be detected with aid of the sensitive Molisch reaction in about half of the cases examined. As the slightest contamination with filter paper leads to a positive result when this delicate test is employed, such an eventuality must be taken into account in judging these results.

It had previously been found impossible to remove the fat entirely when the protein was coagulated in an aqueous medium. The presence of fat could not, however, be detected in the protein residuum obtained by the aid of the present modified technique. The method employed to ascertain this fact was that of Gephart and Csonka<sup>4</sup> with employment of 0.01 N solutions and control of all reagents. *The conclusion may therefore be drawn that, contrary to the generally accepted view, it is possible to free muscle completely of fatty substances by extraction with use of the analytical procedure here described.* The finding of a residuum of fat in muscle extracted even for weeks has been variously reported in the past. This result is due to the methods used. Usually air- or vacuum-dried muscle was extracted with ether. Such material is so inspissated that it is extremely resistant to solvents. Inadequacies in older methods of fat determinations probably represent an additional factor contributing to such results.

The preparations of protein obtained on completion of the analyses are very light in color. It seems scarcely possible that this degree of pigmentation can represent a very appreciable cause of inexactness.

Another possible source of error remains to be discussed, the effect of extraction and the necessary mechanical manipulation on the filter paper weighed with the protein. Two Schleicher and Schull No. 597 papers, 12.5 cm. in diameter, those commonly used by us in this method, were dried to constant weight and then treated precisely as called for in carrying out the analytical procedure except that the muscle was omitted. The weight lost was 0.6 and 0.3 mg. respectively. The error from this source is therefore inconsequential.

<sup>4</sup> Gephart, F. C., and Csonka, F. A., *J. Biol. Chem.*, 1914, xix, 521.

In the previous study considerable time had already been devoted to determining the duration of the Soxhlet extraction required for the complete removal of the fatty substances. In the hope, however, that the preliminary treatment with alcohol as now employed would shorten this period, suitable control experiments were carried out in establishing the accuracy of the present modification. The amount of solids present in the extract was ascertained at intervals, the same precautions being observed as previously.<sup>1</sup> It was found that all but a few mg. of alcohol-soluble material is removed from the protein within 6 hours. This residuum of fatty substances is, however, extracted but slowly, so that a total of 18 hours as compared to 24 previously is found necessary to insure complete extraction. In these controls the ordinary type of Soxhlet apparatus and an electric hot plate were employed. As the length of time required for the complete removal of all extractives varies to some extent according to the amount of extractives, especially fat, present, also with the individual apparatus and precise temperature employed, it is advisable in very exact work to ascertain this point in advance by control of the extract as indicated. An 18 hour extraction as described in detail below represents an entirely safe margin, even when considerable fat is present in the muscle.

We have found that the use of ether is attended by no obvious advantage over alcohol. All ether-soluble substances are removed by the alcohol. This was established by a further series of control experiments which are omitted for the sake of brevity.

*Accuracy of the Modified Method.*—In summarizing the various experiments made to this end, it is believed that consideration has been given to all apparent sources of error which can be controlled. The protein values found are influenced to the average extent of  $-0.15$  by loss of material of protein nature in the extracts,  $+0.05$  by the ash, and  $\pm 0.15$  per cent in duplicate analyses. The method properly carried out may therefore be regarded as accompanied by a total average error probably not exceeding a few tenths of 1 per cent.

#### *Improved Method in Detail.*

The fresh muscle is freed from all adherent fat and connective tissue, passed through a meat grinder, and thoroughly mixed.

About 10 gm. are weighed by difference into a beaker from a weighing glass provided with a ground glass lid. 50 cc. of 95 per cent alcohol are added and the contents of the beaker heated with stirring until the alcohol boils. The liquid is then decanted through an ordinary round filter of 12.5 cm. diameter, which has previously been extracted with alcohol and ether, dried, and weighed. This treatment of the protein with alcohol is once repeated. The coagulated muscle is next extracted in a similar manner with 400 cc. of boiling water in four portions, and then brought quantitatively on the filter. The filter is now carefully folded about the protein material, which is gently inserted into an extraction hull and extracted 3 hours in an ordinary Soxhlet apparatus with 95 per cent alcohol. The 95 per cent alcohol is then replaced by absolute alcohol and the extraction continued for a period of 15 hours. Care must be taken that the filter projects beyond the upper level attainable by the solvent, which must completely surround the protein. After completion of the extraction the filter with the pure protein is removed from the apparatus, dried to constant weight at 105° in a weighing glass provided with a ground glass lid, and the previously ascertained weight of the filter paper deducted.

As 10 gm. muscle samples have been used in the control analyses, this amount is adhered to in the above description. In view of the small error found, the employment of 5 gm. of muscle, with correspondingly smaller amounts of alcohol for coagulation and water for extraction, is permissible. It is indeed an advantage, because the time required for drying the protein and probably also for its Soxhlet extraction is somewhat diminished.

The *non-protein substances* are determined when required, by deducting the per cent of protein found from that of the total solids, which are estimated as described in the earlier communication.<sup>1</sup>

With the help of this method, we have detected a considerable error in the usual modes of estimation of the amount of protein present in muscle. This subject is treated in the note immediately following the present article.<sup>5</sup>

<sup>5</sup> Attention has also been called to these studies in a recent paper read before the Society for Experimental Biology and Medicine (*Proc.*, 1916, xiii, 83).



## THE PROTEIN CONTENT OF MUSCLE.

By N. W. JANNEY.

(From the Chemical Laboratory of the Montefiore Home and Hospital for Chronic Invalids, New York.)

(Received for publication, April 10, 1916.)

An exact knowledge of the composition of animal muscle is of considerable importance for the science of nutrition. Though the proteins represent the most important muscle constituents, the usual means employed to determine the amount of these substances present remain rather inaccurate. Thus the amount of protein in muscle is still most commonly estimated by multiplying the total nitrogen by the classical protein factor 6.25. This mode of calculation is used in most of the standard food analyses tables, such as published by König,<sup>1</sup> Atwater and Bryant,<sup>2</sup> Atwater,<sup>3</sup> and Lusk.<sup>4</sup> On the protein values so obtained are based various metabolic data including calculations of the caloric values. Although this method of estimating muscle protein is generally known to be a faulty one, its inadequacies may still be alluded to in the hope of lessening its present extensive employment. The protein factor 6.25 is based on acceptance of 16.00 per cent as the nitrogen content of muscle proteins. Although many of the older nitrogen analyses of muscle proteins do give values averaging about 16.00 per cent, the muscle protein preparations then analyzed were of very dubious purity. More recently Osborne and his coworkers have reported analyses of carefully prepared animal muscle proteins which are considerably higher than 16.00 per cent. Similar analytical results have also been obtained in the writer's laboratory for pure muscle proteins. The nitrogen content was found to be

<sup>1</sup> König, J., *Chemie der Menschlichen Nahrungs- und Genussmittel*, Berlin, 4th edition, 1910, iii, 252.

<sup>2</sup> Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Bull.* 28, 1906.

<sup>3</sup> Atwater, W. O., *U. S. Dept. Agric., Farmer's Bull.* 142, 1906.

<sup>4</sup> Lusk, G., *The Elements of the Science of Nutrition*, Philadelphia, 2nd edition, 1909.



rather constant for various species of higher animals, ranging from 16.2 to 16.7 per cent in many analyses. In view of this work the protein factor 6.25 can no longer be considered accurate. Moreover, a very considerable error is introduced by using the total nitrogen of muscle in this mode of calculation of the protein, for our analyses have shown that about 13 per cent of the total nitrogen is combined in non-protein substances in muscle. The amount of protein obtained is greatly increased in consequence.

A further source of confusion and error is the loose application of the term "protein" to include practically all protein and "nitrogenous non-protein substances" found in animal muscle. Thus Atwater and Bryant, who adopt this nomenclature, calculate the number of calories obtainable from the proteins of meats from protein in this sense. As extractive substances of non-protein nature are here reckoned in, the result must be inaccurate in the number of calories ascribed to the meat protein.

Another much used indirect method for estimating the amount of muscle protein is that "by difference." According to this plan the total water-free substance is ascertained; and from this value the solid material found in the ether extract of the dried muscle, together with the ash, is subtracted. The result is designated as protein and varies but slightly from that obtained by multiplying the total muscle nitrogen by the factor 6.25. This correspondence is, however, no criterion of accuracy, for nearly the same sources of error are present in both procedures. The "protein" as thus ascertained from anhydrous muscle material includes also the non-protein extractives aside from those soluble in ether. In the article immediately preceding mention has also been made of the fact that it is very difficult to free dried muscle entirely of fatty substances by means of ether extraction. Any residual amount of fat thus remaining in the extracted protein would serve to increase the protein content obtained by this mode of calculation. This presents an additional source of inaccuracy. Lastly the carbohydrate content of muscle is likewise reckoned as protein by this procedure. A further small but appreciable error is thus introduced.

To enter into a discussion of various other methods proposed for the estimation of muscle proteins is scarcely advisable in view of the fact that none have come into very general use. The chief

reason for this is that these procedures are of questionable accuracy in nearly all cases.

These considerations have led the writer to devise a new method for the determination of the muscle proteins which permits of a rather exact determination of these substances. The improved technique of this method is described in the preceding article.

In the table are arranged for comparison protein values taken from Atwater and Bryant's food analyses obtained by multiplication of the total muscle nitrogen by the factor 6.25, as well as "by difference," as described above. Average results of a number of the writer's analyses appear also. All the analyses refer to the same kind of muscle as indicated. In the majority of cases

*Comparison of Calculated and Actual Amounts of Protein in Muscle.*

Species	Total nitrogen in muscle	Protein		
		N $\times$ 6.25	By difference	Average of analyses according to the writer
	per cent	per cent	per cent	per cent
Chicken.....	3.09*	19.3	19.0	16.6
Fish (halibut)...	2.98*	18.6	18.4	16.5
Ox .....	3.46	21.6	21.5	16.6
Rabbit.....	3.39	20.8		16.3
Cat.....	3.38	21.1		17.8
Dog .....	3.25	20.2		17.4
Man.....	3.15	19.7		16.4

\*Calculated from Atwater and Bryant's protein values. The remaining nitrogen analyses are by the writer.

the nitrogen was estimated in the same sample of muscle for which the protein was determined according to the writer's method. *The amount of protein calculated is seen to exceed that determined by actual analysis by about 15 to 20 per cent in nearly all cases. Continuance of the use of these usually employed methods of calculating muscle protein should therefore be discouraged.*

In order to put calculations of the caloric values of meat proteins on a more exact basis, the calories yielded by the pure muscle protein obtained in the analytical method should be determined. As it is possible to make preparations of meat extractives without

appreciable loss,<sup>5</sup> the caloric value of such preparations can also be obtained and correspondingly allowed for in metabolic work of this description.

The table also brings out the interesting fact that the actual protein content of muscle is usually quite constant for various species, even those as far separated zoologically as man and fish. In wasting diseases among human beings, however, values as low as twelve have been recorded. It seems possible that in such muscular disorders as myasthenia gravis the amount of protein muscle substance present might be found to bear a definite relation to the specific muscular seat of the disease.

<sup>5</sup> Janney, N. W., and Csonka, F. A., *J. Biol. Chem.*, 1915, xxii, 195.

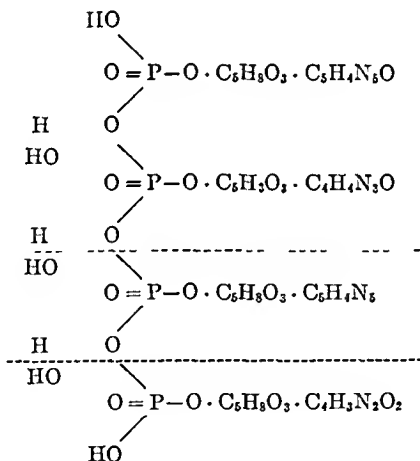
# THE PARTITION OF PHOSPHORUS IN THYMUS NUCLEIC ACID.

By HILDEGARDE C GERMANN.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University, Baltimore )

(Received for publication, April 4, 1916 )

From a study of the optical changes which are brought about in yeast nucleic acid by the action of ferments, Levene and Medigreceanu<sup>1</sup> concluded that in the first phase of the enzymatic decomposition of the substance its four component nucleotides are produced:



Levene and Jacobs<sup>2</sup> showed also that yeast nucleic acid is initially decomposed in the same way by hydrolysis with dilute sulfuric acid, but that the two purine mono-nucleotides so formed are further decomposed, by prolonged acid hydrolysis, into phos-

<sup>1</sup> Levene, P. A , and Medigreceanu, F , *J Biol Chem* , 1911, ix, 389

<sup>2</sup> Levene, P. A , and Jacobs, W A , *Ber chem Ges* , 1911, xlv, 1027

phoric acid *d*-ribose and purine base, while the two pyrimidine mono-nucleotides remain unaltered.

Presumably any ribose nucleotide will decompose under the same conditions into its component mono-nucleotides, and by continued boiling with dilute acid the purine mono-nucleotides so formed will surely part with their phosphoric acid, but the pyrimidine mono-nucleotides will not. Therefore, a comparison of the easily split phosphoric acid with the total phosphoric acid obtainable from a compound nucleotide will give the ratio of its purine groups to its total nitrogenous groups, and the numerical index of the nucleotide may be ascertained.

Jones and Riley<sup>3</sup> have recently described a method by which this phosphoric acid ratio can be quickly and accurately determined. The results obtained may be interpreted from the following considerations.

A tetra-nucleotide (such as yeast nucleic acid) which contains two purine groups and two pyrimidine groups will give off half of its phosphoric acid by mild hydrolysis with sulfuric acid. So also will a di-nucleotide that contains one purine group and one pyrimidine group. But in such cases it is easy to show that the di-nucleotide contains but one purine group or even that it produces twice as much of the purine compound as could be obtained from a tetra-nucleotide.

A purine mono-nucleotide will give off all of its phosphoric acid while a pyrimidine mono-nucleotide will give off none of its phosphoric acid by mild acid hydrolysis. A tri-nucleotide would offer an excellent opportunity for the application of this method. If such a compound should contain one pyrimidine group and two purine groups it would give off two-thirds of its phosphoric acid; but if it should contain two pyrimidine groups and one purine group it would give off only one-third of its phosphoric acid by mild acid hydrolysis.

In case a nucleotide is contaminated with other substances (nucleosides, purine bases, or any compounds that do not produce phosphoric acid) its total phosphorus will be low but the numerical index of the nucleotide will not be affected by the presence of the impurity.

<sup>3</sup> Jones, W., and Riley, C., *J. Biol. Chem.*, 1916, xxiv, p. iii.

In case one is dealing with mechanical mixtures of different nucleotides, the numerical index found will not be a whole number. This was found true of a preparation that had been obtained by hydrolysis of yeast nucleic acid with ammonia at 115°. There was some reason for believing the substance to be a tri-nucleotide; but it is in fact a mixture of guanylic acid with a purine-pyrimidine di-nucleotide.<sup>4</sup> A dozen or more experiments with different preparations of the substance gave numerical indices from 2.35 to 2.68.

The application of this method to the animal nucleic acids can scarcely be without interest.

Thymus nucleic acid differs at two points from yeast nucleic acid as far as its fundamental groups are concerned. The carbohydrate group of thymus nucleic acid is a hexose group; that of yeast nucleic acid is a pentose group. The pyrimidine groups of thymus nucleic acid are thymine and cytosine groups; those of yeast nucleic acid are uracil and cytosine groups. According to Levene and Jacobs<sup>5</sup> both nucleic acids are tetra-nucleotides made up of two purine mono-nucleotides and two pyrimidine mono-nucleotides, but in the structure of the two nucleic acids their component mono-nucleotides are bound to one another at different points of juncture. It seems probable that the initial stage of acid hydrolysis would be the same in both cases; *viz.*, a splitting of the tetra-nucleotide into four mono-nucleotides; and one would suspect (after the analogy of yeast nucleic acid) that the two purine nucleotides of thymus nucleic acid would give up phosphoric acid by further acid hydrolysis while the pyrimidine nucleotides would not. One would therefore expect to find a tetra-nucleotide index. But this is not the case; the phosphorus values obtained lead sharply to a tri- or hexa-nucleotide index.

#### EXPERIMENTAL.

The sodium salt of thymus nucleic acid was prepared by the method of Neumann<sup>6</sup> and in order to remove a small amount of free phosphoric acid, the material was dissolved in hot water and treated with enough caustic soda to make a 1 per cent alkaline

<sup>4</sup> Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93.

<sup>5</sup> Levene and Jacobs, *J. Biol. Chem.*, 1912, xii, 411.

<sup>6</sup> Neumann, *Arch. f. Physiol., Suppl.*, 1899, 552.

solution. The warm solution was filtered from a little gelatinous phosphate (using a hot water funnel) and, after acidifying with acetic acid, the pale yellow fluid was warmed and poured into a large excess of alcohol. The precipitated sodium nucleate was washed by decantation with absolute alcohol and allowed to dry in a sulfuric acid desiccator.

Ten portions of the nucleic acid were weighed out. Two of these, intended for duplicate determinations of phosphoric acid were received into 500 cc. digestion flasks, and the other eight portions, which were to be used for determinations of partial phosphoric acid, were received into 100 cc. Erlenmeyer flasks which had been provided with corks and condensing tubes.

The determinations of total phosphoric acid were made as follows: The material was treated with a mixture of 5 cc. of concentrated sulfuric acid and 5 cc. of concentrated nitric acid, and boiled gently until all red fumes had been driven away and the liquid had become perfectly colorless. After cooling, 5 cc. of concentrated nitric acid were added and the product was again heated. As a third addition of nitric acid did not cause the production of brown fumes, the material was assumed to be sufficiently oxidized. The product was diluted to about 50 cc. with water, treated at the boiling point with 10 gm. of ammonium nitrate and an excess of 3 per cent ammonium molybdate, and the yellow ammonium phosphomolybdate was converted by the usual method into crystalline magnesium ammonium phosphate. The latter substance was allowed to dry for 48 hours at room temperature and was weighed.

The determinations of partial phosphoric acid were made as follows: The eight portions of nucleic acid were treated with 5 per cent sulfuric acid (20 cc. per gm. of nucleic acid) and after solution had been effected by heating, the condensing tubes were inserted and the flasks were submerged in a boiling water bath. Care was taken that the level of the liquid in the flasks was not below the level of the water in the water bath. The flasks were thus heated for  $\frac{1}{2}$  to 7 hours. As each flask was removed at the end of its time interval, the contents were made roughly alkaline with concentrated ammonia and, without waiting for the precipitation of guanine, the phosphoric acid was carefully precipitated with magnesia mixture. On standing over night the guanine and magnesium ammonium phosphate had settled sharply, leaving a perfectly clear, easily filterable fluid. This step of the procedure is absolutely necessary, because if an attempt be made to remove the guanine first (by precipitation with ammonia, as is easily done with the hydrolytic products of yeast nucleic acid), the guanine will be thrown out in so finely divided a form that it cannot be removed either by filtration or by centrifugation. But when the guanine and phosphoric acid are precipitated together, the fluid which contains the combined phosphoric acid can easily be filtered off.

The precipitate consisting of a mixture of guanine and magnesium ammonium phosphate was put into a 500 cc. Kjeldahl digestion flask and heated with a mixture of 5 cc. of concentrated nitric acid and 5 cc. of concentrated sulfuric acid until all organic matter had been destroyed. The determination of phosphoric acid in the oxidized mixture was then made by precipitation in turn as ammonium phosphomolybdate and magnesium ammonium phosphate, as described for the determination of total phosphoric acid. The results are given in Table I.

TABLE I.

Nucleic acid used.	Time.	Magnesium ammonium phosphate $MgNH_4PO_4 \cdot 6H_2O$ .			
		Obtained.	Per gm. of nucleic acid	Correction.	Remainder.
gm.	hrs.	gm.	gm.	gm.	gm.
0.7660	$\frac{1}{2}$	0.1015	0.132	0.006	0.126
0.7523	1	0.1303	0.173	0.011	0.162
0.6010	2	0.1326	0.221	0.022	0.199
0.7418	3	0.1774	0.239	0.033	0.206
0.7227	4	0.1856	0.257	0.044	0.213
0.7443	5	0.1977	0.265	0.055	0.210
0.5423	6	0.1506	0.277	0.066	0.211
0.9087	7	0.2549	0.280	0.077	0.203
0.4994	Total.	0.3027	0.606		
0.6245	"	0.3791	0.607		

In order that the significance of these results may be more apparent they have been constructed into a curve, one of whose coordinates is time, and the other, amount of magnesium am-

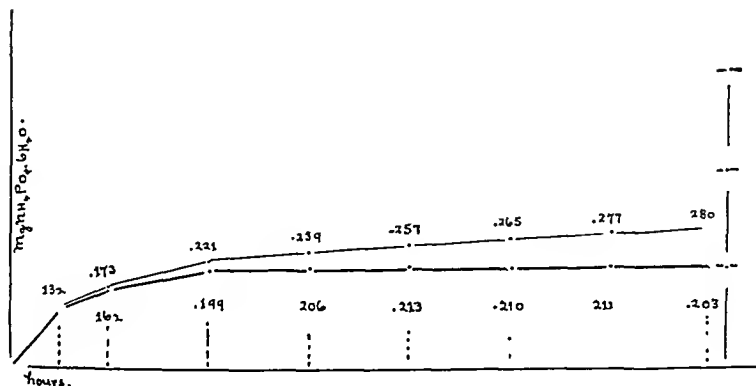


FIG. 1.



monium phosphate. The linear units have been arbitrarily chosen so as to bring out curvature as prominently as possible.

The principle of construction is that which Jones and Riley<sup>3</sup> have described in connection with yeast nucleic acid. The ordinates of points on the upper curve represent the quantities of magnesium ammonium phosphate actually obtained. This curve turns at the end of the 2 hour period and becomes a straight line slightly inclined to the axis of X, and its inclination shows that an amount of phosphoric acid corresponding to about 11 mg. of ammonium magnesium phosphate has been set free per hour. This amount was subtracted from each ordinate and the remainders were used as ordinates for the lower curve which ends in a straight line parallel to the axis of X and represents the amount of easily split phosphoric acid.

It will be noted that certain points do not fall exactly on the curve. This is due to uneven heating. It has been observed that when a flask dips too far below the level of the water in the water bath the experiment gives a point above the curve.

Nevertheless, the results as given are sufficiently accurate to show that of the phosphoric acid combined in thymus nucleic acid, one-third is easily split and two-thirds are firmly bound. This leads definitely to the conclusion that the number of phosphorus atoms in the molecule of thymus nucleic acid is divisible by three.

It is of interest to know whether this easily split phosphoric acid corresponds to purine groups, as is the case with yeast nucleic acid. But the amount of purine bases liberated from thymus nucleic acid by mild acid hydrolysis cannot easily be determined. The presence in the product of the undecomposed parts of the molecule interferes and there is danger of liberating purine bases in attempting to determine those already free. The matter offers considerable experimental difficulty but is being examined.

Experiments are also in progress with other animal nucleic acids.

## THE DETERMINATION OF CREATINE IN MUSCLE. III.

By LOUIS BAUMANN AND THORSTEN INGVALDSEN.<sup>1</sup>

(From the Chemical Research Laboratory, Department of Internal Medicine,  
State University of Iowa, Iowa City.)

(Received for publication, April 10, 1916.)

Muscle creatine is now determined colorimetrically after conversion into creatinine. As the Jaffé color test is not specific, it is questionable whether creatinine alone is responsible for this reaction. For this reason an apparent increase in the creatine content of muscle in feeding and perfusion experiments may in reality be due to some other substance. It therefore seemed desirable to develop a method for the isolation of the creatinine from muscle extract, preferably as the difficultly soluble potassium picrate salt. The creatinine content of the precipitate could then be estimated colorimetrically. Morris<sup>2</sup> has applied a somewhat similar procedure to the urine.

After considerable preliminary work the following technique was adopted. Approximately 5 gm. of hashed and well mixed muscle are weighed on the analytical balance and extracted according to Janney and Blatherwick.<sup>3</sup> After adding 5 cc. of 5 N hydrochloric acid and a pinch of granulated lead the water-clear extract is evaporated to dryness, at first over the flame and finally on the water bath.<sup>4</sup> The residue is taken up in hot water and filtered through a small filter. The washings and filtrate should not exceed 35 cc.

<sup>1</sup> The experimental data are taken from a dissertation submitted by Thorsten Ingvaldsen as a partial requirement for the degree of Master of Science, State University of Iowa, Iowa City.

<sup>2</sup> Morris, J. L., *J. Biol. Chem.*, 1915, xxi, 201.

<sup>3</sup> Janney, N. W., and Blatherwick, N. R., *J. Biol. Chem.*, 1915, xxi, 567.

<sup>4</sup> Benedict, S. R., *J. Biol. Chem.*, 1914, xviii, 191. We have found, in corroboration of Benedict's statement, that this method is the most rapid for the conversion of urinary creatine into creatinine. It is accurate in the absence of sugar.

monium phosphate. The linear units have been arbitrarily chosen so as to bring out curvature as prominently as possible.

The principle of construction is that which Jones and Riley<sup>3</sup> have described in connection with yeast nucleic acid. The ordinates of points on the upper curve represent the quantities of magnesium ammonium phosphate actually obtained. This curve turns at the end of the 2 hour period and becomes a straight line slightly inclined to the axis of X, and its inclination shows that an amount of phosphoric acid corresponding to about 11 mg. of ammonium magnesium phosphate has been set free per hour. This amount was subtracted from each ordinate and the remainders were used as ordinates for the lower curve which ends in a straight line parallel to the axis of X and represents the amount of easily split phosphoric acid.

It will be noted that certain points do not fall exactly on the curve. This is due to uneven heating. It has been observed that when a flask dips too far below the level of the water in the water bath the experiment gives a point above the curve.

Nevertheless, the results as given are sufficiently accurate to show that of the phosphoric acid combined in thymus nucleic acid, one-third is easily split and two-thirds are firmly bound. This leads definitely to the conclusion that the number of phosphorus atoms in the molecule of thymus nucleic acid is divisible by three.

It is of interest to know whether this easily split phosphoric acid corresponds to purine groups, as is the case with yeast nucleic acid. But the amount of purine bases liberated from thymus nucleic acid by mild acid hydrolysis cannot easily be determined. The presence in the product of the undecomposed parts of the molecule interferes and there is danger of liberating purine bases in attempting to determine those already free. The matter offers considerable experimental difficulty but is being examined.

Experiments are also in progress with other animal nucleic acids.

A comparison of the figures obtained by this procedure with those found by the direct methods of Janney and Blatherwick<sup>3</sup> or Baumann<sup>7</sup> shows close agreement.

On several occasions the picrate was decomposed with acid, the picric acid removed, and the nitrogen content of the solution determined. Here again the amount of nitrogen found agreed closely with the calculated quantity. This last step may become necessary when the presence of a substance other than creatinine which also forms an insoluble picrate is suspected.

#### EXPERIMENTAL.

##### *Creatinine Can Be Quantitatively Precipitated with Picric Acid and Potassium Picrate.*

To 20 cc. of a 0.1 N hydrochloric acid solution containing 20 mg. of creatinine, 2 cc. of 5 N sulfuric acid solution were added. The solution was neutralized and further treated as described above. This experiment was carried out in triplicate.

Found: 19.97, 19.97, and 19.97 mg. of creatinine.

##### *Conversion of Creatine into Creatinine.*

At the outset it was necessary to select a method of conversion which would avoid the use of an excess of acid.<sup>8</sup> Conversion by the autoclave method of Myers<sup>9</sup> was first tried. 2 cc. of 5 N sulfuric acid were added to the concentrated muscle extract (about 10 cc.) and this was then heated in the autoclave at 116–117° for 40 minutes. The figures obtained after precipitation are tabulated below. The direct controls were carried out according to the methods of Janney and Blatherwick or Baumann.

<sup>7</sup> Baumann, L., *J. Biol. Chem.*, 1914, xvii, 15.

<sup>8</sup> An excess of sodium picrate was found to be undesirable.

<sup>9</sup> Benedict, F. G., and Myers, V. C., *Am. J. Physiol.*, 1907, xviii, 397.  
Benedict, F. G., *J. Biol. Chem.*, 1914, xvii, 363.

*Conversion by Myer's Method.*

Dog muscle		Beef muscle.	
Direct.	Precipitation.	Direct.	Precipitation.
mg.	mg.	mg.	mg.
373	352	485	474, 463, 464, 480, 487, 486, 467, 468, 475, 470, 478
		485	469
		412	386, 375, 373, 385, 390, 390, 383, 382, 384,* 385
		360	354, 343, 362, 367
		443	411, 418, 408, 417, 429, 414, 423, 436
		369	328, 318, 355, 356

\* Determined directly after conversion without precipitation.

Acceptable results were obtained when the creatine was converted by boiling the concentrated extract (about 10 cc.) obtained from approximately 5 gm. of muscle, with 2 cc. of 5 N sulfuric acid for 3 hours under a reflux.

Beef muscle.	
Direct.*	Precipitation.
mg.	mg.
355	353, 357, 351, 355**
456	461, 455, 456**
464	458, 467, 459, 472, 461,** 474**

\*Determined according to Janney and Blatherwick.

\*\*Determined directly after conversion without precipitation.

This method was then compared with the autoclave method on a solution of pure creatine. 10 cc. of solution contained 19.76 mg. of pure anhydrous creatine.

Found: (a) 19.6 mg. 10 cc. boiled for 3 hours with 2 cc. of 5 N sulfuric acid.

(b) 19.14 " 10 cc. autoclaved with 1 cc. of 5 N sulfuric acid for 40 minutes at 117°.

(c) 18.92 " Same as (b).

(d) 19.46 " Same as (b) except that 2 cc. of acid were used

(e) 19.62 " Same as (d).

Conversion, preliminary to precipitation, was finally carried out according to the method of Benedict.<sup>4</sup> It appeared to be the simplest method and as accurate as any of the others.

*Conversion by the S. R. Benedict Method.*

Direct.*	Direct.**	Direct.***	Precipitation.	Muscle.
mg.	mg.	mg.	mg.	
449		445	433	Beef XVI
426		404	405	" XVII
		401	403	" XVII
		402	401	" XVII
450		428	422	" XVIII
		419	433	" XVIII
		421	427	" XVIII
	509	532	490	" XIX
		531	494	" XIX
	476	479	479	" XX
		479	483	" XX
			483	" XX
	417	431	431	Dog XXI
		442	431	" XXI
	476	515	522	Muskrat XXII
			503	" XXII
481	454	480	481	Beef XXIII
			479	" XXIII

\*Baumann method.

\*\*Janney and Blatherwick method.

\*\*\*S. R. Benedict method without precipitation.

During the early period of this work it was occasionally necessary to purify the concentrated muscle extract with colloidal ferric hydroxide. The following experiment indicates that the iron precipitation did not remove creatinine from the solution. A converted muscle extract was diluted to a definite volume and the creatinine content of an aliquot portion determined. The remainder was accurately neutralized, precipitated with the colloidal iron solution, and the creatinine estimated in the filtrate.

Result: With iron 407, without iron 407 mg.

*Analysis of the Precipitated Picrate.*

The concentrated extracts from three 5 gm. muscle samples were converted, then united and precipitated with picric acid and

potassium picrate in the usual way. 4 gm. of picric acid and 0.8 gm. of potassium picrate were employed for this purpose. After 12 hours in the cold, the supernatant liquid was decanted through a Gooch crucible which had previously been washed with saturated picric solution. The bulk of the precipitate which had remained in the beaker was then washed with 30 cc. of ice-cooled saturated picric acid solution by decantation. The crucible was then connected with a clean flask and 10 cc. of 45 per cent sulfuric acid solution were poured on the precipitate in the beaker, and then filtered through the crucible. This process was repeated three times with fresh 10 cc. portions of acid. The first part of the filtrate was returned to the crucible to remove a small quantity of picric acid which had crystallized. The picric acid residue was finally washed with two 10 cc. portions of 5 N sulfuric acid solution. The remainder of picric acid, dissolved in the filtrate, was removed by thoroughly shaking with ether. This was repeated until the ether was colorless. The ether was removed with a pipette connected with a filter pump. The nitrogen in the picric acid-free solution was then determined according to Kjeldahl. It was necessary to boil 1 hour after the fumes began to be given off. The following results were obtained.

Beef muscle.	
Direct.	Calculated from the N found.
mg.	mg.
417	417
418	386, 403
482	481, 495
412	416

#### CONCLUSIONS.

1. Creatinine may be quantitatively precipitated from muscle extracts by means of picric acid and potassium picrate.
2. The values obtained by the precipitation method agree with those found by the direct methods of Janney and Blatherwick, S. R. Benedict, and Baumann.

# IS AUTOLYSIS AN AUTOCATALYTIC PHENOMENON? AN INTERPRETATION.

By H. C. BRADLEY.

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Madison.)

(Received for publication, March 27, 1916.)

In a recent paper bearing the above title Morse<sup>1</sup> shows that the curve of hydrogen ion concentration in an autolyzing tissue is of the logarithmic type and resembles in a general way the curve of proteolysis in the same tissues. This confirms the common observation that a tissue grows more distinctly acid to litmus as it autolyzes, and is in agreement with the observations of Kikkoji<sup>2</sup> and others regarding the formation of lactic acid in autolyzing tissues.

From these interesting data Morse draws the conclusion: "*Autolysis is an autocatalytic phenomenon, the products of digestion entering into the reaction as true catalyzers.*" To be autocatalytic the process must be, as Morse states earlier in the paper, one "*in which the process of enzyme action is accelerated by the products formed during the action of the enzyme and in consequence of it.*" But since *autolysis* is synonymous with *proteolysis* throughout the paper, and the enzyme in question is a proteolytic one, the conclusion would appear to affirm that the products of proteolysis—peptones, amino-acids, etc.—accelerate the tissue proteolysis. This conclusion we do not believe is intended by Morse.

In another place the statement is made that "*developing acidity*" induces "*greater and greater acceleration in the digestion rate.*" None of the curves which Morse cites to illustrate this, including curves of his own construction, show the slightest indication of acceleration in the rate of digestion. The curves rise most rapidly at the beginning and proceed at a diminishing rate of change until

<sup>1</sup> Morse, M., *J. Biol. Chem.*, 1916, xxiv, 163.

<sup>2</sup> Kikkoji, T., *Z. physiol. Chem.*, 1907, liii, 415.



equilibrium is attained. At this point—the maximum extent of digestion—the rate of change is zero.

It appears fair to conclude, therefore, that Morse does not mean exactly what he states in his conclusions in this paper, but that some confusion of ideas has occurred in his interpretation of his results. A part of this confusion appears to arise from a failure to differentiate sharply between the *rate of change* in the reaction and the *extent of change* or point of equilibrium. If the rate of change did actually increase, as Morse incorrectly asserts in the sentence quoted above, the reaction would indeed be an autocatalytic one. Since it does not, we believe the term is misleading and will only cause confusion if left unchallenged.

What we assume Morse intends to convey in this paper is this. *During the complex of reactions broadly termed autolysis, acids are known to appear. Their formation causes an increase in the acidity of the tissue as shown by the hydrogen ion concentration. Acidity renders the proteolytic enzymes active and is without effect upon the substratum. Proteolysis results. The products of acid formation thus activate the enzymes causing proteolysis, and in this sense a tissue considered as a whole, starts itself digesting its own proteins.*

Even assuming the above statement of the fact of activation to be correct, this is not autocatalysis unless the acids are produced by proteolytic enzymes, and of this no evidence is given. There is no evidence in the paper to substantiate the fact of enzyme activation which Morse assumes. Indeed the experiment is not calculated to determine this point, since the activity of an enzyme can only be judged by a digestion experiment. In spite of this lack of direct evidence, and because the curve of hydrogen ion concentration shows no sudden break during autolysis, Morse says "we should seek the simpler explanation, that the acidity developed acts catalytically, activating the enzyme in much the same manner that the hydrochloric acid of gastric digestion activates; for there is nothing to indicate a sudden alteration in the  $H^+$  content in the curves." The lack of sudden alteration in the  $H^+$  content of autolyzing tissue is merely proof that the proteins are still unsaturated with acid and are serving like buffer salts into which considerable acid may be poured before a sudden alteration of the  $H^+$  will occur. Strong HCl may be added to a 20 per cent liver

digest to a titratable strength of 0.04 N without increasing the  $H^+$  content above that expressed by about  $P_H$  6.00. This is far more acid than is formed in the normal control digest and produces a far more rapid and complete proteolysis.

There is no essential difference between development of acidity by the formation of lactic and fatty acids in a digest and the introduction of such acids artificially from without. The same mechanism, determining the rate and extent of digestion, must be operative in both cases. Morse believes this mechanism to be activation of the proteolytic enzymes alone, at least in the normal tissue. On the other hand, we have presented data<sup>3</sup> which throw some doubt on activation as of major importance in this reaction. It is entirely possible that an enzyme such as the  $\beta$  protease found by Hedin<sup>4</sup> in the spleen, active only in acid media, is also present in the liver. Whatever the nature of the enzyme we know that it is active in a  $H^+$  concentration between  $P_H$  7.3 and 6.7, since most livers show proteolysis within these concentrations. The enzyme is thus active at a fairly low  $H^+$  level. The fact that casein and peptone digest no faster in an acidified digest than in a normal control indicates that further additions of acid to the already active enzyme have little or no effect upon its activity. Yet such additions of acid have a tremendous effect in increasing the rate and extent of digestion of the liver proteins. This is, we feel, rather direct proof of alteration of the mass of the substratum.

The data which Morse has presented and which we assume to be correct may therefore be interpreted in quite a different manner without doing violence to the facts developed by previous work in the field and without involving confusion.

1. At a  $P_H$  of about 7.00  $\pm$  the liver protease is active as shown by the fact that some proteolysis goes on at that level.

2. Acid develops in the autolyzing mixture, rapidly at first, reaching its maximum concentration in about 3 days.

3. Accompanying this increased acidity and caused by it, is a corresponding change in the protein substratum of the tissue. If much acid is produced the extent of digestion will be large; if little is produced digestion will reach equilibrium at a lower level.

<sup>3</sup> Bradley, H. C., *J. Biol. Chem.*, 1915, xxii, 113.

<sup>4</sup> Hedin, S. G., *J. Physiol.*, 1904, xxx, 155.

4. The mass of substratum will determine the point of equilibrium, but the amount of acid will determine the mass of substratum.

5. There is nothing in the data and curves of autolysis thus far presented in the literature to suggest an autocatalytic reaction.

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# A NEW SALT OF URIC ACID AND ITS APPLICATION TO THE ANALYSIS OF URIC ACID AND PHENOL.

PRELIMINARY PAPER.

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(Received for publication, April 17, 1916.)

Some time ago the writer had occasion to use the colorimetric analysis of uric acid as a routine procedure in a large number of determinations. The small amount of material available, and the large amount of polyphenols or other interfering substances<sup>1</sup> present emphasized certain difficulties of the method. From time to time since then the failure of an analysis without any apparent cause has compelled interest in those factors which may interfere with a perfect result. Probably the most constant element of difficulty in this method is, as in all work with uric acid, very closely associated with its marked insolubility. But the effect of the presence of various salts, even in very small amounts, upon the colorimetric determination, is noticeable. The observations so far made do not justify a general conclusion as to the nature of the interference with the color development, but there is considerable evidence that it is due to the formation of urates and the uric acid is so removed from the reaction with the phosphotungstic reagent. Whether this is true in all cases is still a question, but the writer believes that he has shown it definitely for the marked interference due to the presence of zinc salts in the uric acid solution during preparation for the color reaction, when the amount of color may be very seriously diminished or entirely absent, or after the development of the color, when fading is much accelerated.

Out of the recognition of such an action of zinc upon uric acid grew the idea that possibly the compound so formed might be

<sup>1</sup> The work referred to was on 24 hour rat urines.

useful in precipitating uric acid. Upon searching the literature no mention of a zinc urate was found. Attempts were made at precipitating it, but with little encouragement. Various zinc salts were added to uric acid and urates, and though precipitates were formed when the solution was made alkaline, there was no evidence that the precipitates consisted of any substance other than zinc hydroxide, carbonate, or phosphate, according to the medium in which the uric acid had been dissolved. Nor did analysis of the filtrates from such precipitates give any indication of the amount carried out of solution, because of the great uncertainty of the value of any results obtained in the presence of such rapid fading as is caused by zinc compounds.

At this point Ganassini's reaction<sup>2</sup> came to my attention. The directions are to add soluble zinc salts to a weakly alkaline solution of uric acid, thus obtaining a white precipitate which turns blue on the filter paper, due to oxidation in the presence of air. By the use of this qualitative test, the writer was able to detect the presence of small amounts of uric acid in considerable volumes of water. This strengthened the belief that uric acid was precipitated by zinc, though it was possible that it was carried down mechanically by the flocculent precipitates of zinc carbonate, zinc hydroxide, or zinc phosphate. In either case, the zinc must evidently be removed from the filtrate if the colorimetric method,<sup>3</sup> or, for that matter, any of the other methods, be used to determine how much uric acid had been removed from solution. This was accomplished by precipitating it as sulfide after acidifying with acetic acid. (This precipitation which is generally so troublesome, was made entirely satisfactory by adding about 0.25 to 0.5 gm. of bismuth carbonate to the hot solution. Then upon saturating with hydrogen sulfide, the finely divided white zinc sulfide is apparently enveloped by the bismuth sulfide, and when enough bismuth is present, the precipitate of mixed sulfides settles from a clear solution. The subsequent filtration is then as easy as that of bismuth sulfide

<sup>2</sup>Ganassini, D., *Bol. Soc. med. chir.*, Pavia, 1908, i; reviewed, *Biochem.* 1908-09, viii, 250.

<sup>3</sup>Nedick, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619. Phosphate-uric acid standard and the KCN substitution for  $H_2S$  as used as modifications of the Folin-Denis method throughout this

alone.) The filtrate was evaporated over a flame, an air current being passed over the surface of the liquid to hasten the evaporation as well as the escape of the hydrogen sulfide. When the volume had been diminished to 10 to 15 cc., the uric acid was determined in the usual colorimetric manner. The results showed conclusively that the uric acid was largely removed from its solution in sodium carbonate, lithium carbonate, or the phosphate mixture of Benedict and Hitchcock<sup>3</sup> by the zinc precipitation. Precipitation from a pyridine solution was also very evident, though the presence of pyridine made the removal of the zinc, and therefore the subsequent determination, less satisfactory.

Attempts were made to obtain the compound of zinc and uric acid for identification. Such efforts have so far proven uniformly unsuccessful. The reason is that the nature of the compound is so characteristically that of other zinc salts, *e.g.*, the phosphate, the carbonate, and the hydroxide, that it cannot be obtained separate from these compounds. The reaction must be neutral, or, better, slightly alkaline to precipitate the zinc-uric acid compound, and this is the condition favoring the precipitation of the zinc in the form of its compound with the alkali used. The same state of affairs exists of course when pyridine or other organic base is the alkali. If the precipitate is then treated with acid or alkali, the uric acid is set free from the compound either in the form of the acid or its alkali salt. This close association with any or all zinc compounds that may be present has stood in the way of every attempt at its separation or purification. This fact, annoying as it has proven, is in itself strong evidence that the compound is zinc urate.<sup>4</sup>

While trying to separate zinc urate in a pure state, the conditions for its most complete precipitation were obtained. They are as follows: Acidify the solution containing uric acid with acetic acid and add 10 per cent zinc acetate solution in a quantity in excess of the amount required to precipitate the phosphates present. Stir well and then add saturated sodium carbonate solution until the reaction is alkaline to litmus. There will be a flocculent precipitate of zinc carbonate and zinc urate (also phosphate if present). If it is desired to determine the amount of uric acid which

<sup>4</sup> Further attempts are being made to obtain the salt in the pure state.

temperatures, it has seemed probable that the precipitation may prove useful in determining uric acid in urine and blood. This is now being investigated by the writer, who hopes soon to publish conditions bearing upon this practical application of the new zinc urate precipitation.

# CHEMICAL AND PHYSICAL ANALYSIS OF BLOOD IN THIRTY NORMAL CASES.

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(Received for publication, April 6, 1916.)

## INTRODUCTION.

Although normal values for various blood constituents and blood serum constants have been published within the last few years by various workers, there is no record where they have all been determined in the same individual and on blood taken at one time. Furthermore, the values given in the literature disagree more or less; this may be due to the different methods in use by various workers, and also to the nature and time of the preceding meal or meals. It was therefore decided to adhere to some fixed standard conditions which could easily be maintained in similar work on patients in the hospital.

*Standard Conditions.*—(1) Breakfast, consisting of one egg, one bread roll with butter, and one cup of coffee with 20 cc. of milk (the regular hospital breakfast). (2) Blood is taken 3 hours thereafter. (3) Determinations are started at once. (4) The same standard methods are always used.

## *Determinations Made.*

*Blood, Chemical.*—Solids, coagulable nitrogen, non-coagulable nitrogen, ammonia nitrogen, urea nitrogen, residual nitrogen, uric acid, creatine, creatinine, chlorides, glucose, fat, cholesterol.

*Blood, Physical.*—Conductivity, hydrogen ion.

*Blood, Biological.*—Red count, white count, corpuscular volume, hemoglobin, Wassermann test.

*Plasma.*—Alkaline reserve, chlorides, nitrogen.

*Serum.*—Specific gravity, freezing point, conductivity, refraction, nitrogen.



*Cases Studied.*

Normal men and women in robust health and in active pursuits (teachers, medical students, laboratory workers, business men, laborers, and others), ranging in ages from 17 to 60 years, volunteered the material on which this study is based. Cases 22 to 26 were hospital patients (simple fracture cases from the surgical service of Professor John A. Hartwell, to whom we are indebted for allowing us to study these cases), examined on the morning of their discharge. Physical examinations and urine analyses were made on these and on all other cases of the series.

*Time of Taking Blood.*—In a preliminary investigation in which normal blood was studied after a 12 hour fast and again 2 or 3 hours after a meal (comprising one egg, one bread roll with butter, coffee, and milk) the values were always found to lie within the normal range. In this series, for convenience, blood was removed 3 hours after a breakfast as above.

*Method of Blood Letting.*—A few drops of 2 per cent novocaine were injected for local anesthesia. A short but large caliber needle was introduced into the median vein, and 75 cc. of blood were allowed to flow out in 3 to 5 minutes. This was collected in sterile, clean, Erlenmeyer flasks, 50 cc. being taken in one containing 0.5 gm. of potassium oxalate to prevent clotting, and 25 cc. in another flask without oxalate. The latter was allowed to stand in the ice box 2 to 3 hours after clotting, preliminary to pouring off serum.

*Blood Counts.*—Red and white counts were made on all cases at the time of phlebotomy. Total counts only are tabulated, differentials having been made in those cases exceeding 9,000 white corpuseles per c.mm., to exclude possible abnormal conditions.

*Hemoglobin.*—This was estimated by Dare's hemoglobinometer,<sup>1</sup> which appears to be quite accurate for the higher readings presented by normals.

*Corpuscular Volume.*—In conjunction with the preceding determinations that of corpuscular volume seems valuable. This observation is made by immediately centrifuging the freshly drawn and well mixed oxalated blood in a calibrated capillary

<sup>1</sup> Dare, A., *Phila. Med. J.*, 1898, vi, 557.

tube. These were made<sup>2</sup> 10 cm. long to be conveniently carried in the jacket of the regular 15 cc. conical centrifuge tubes. A large elastic band is slipped about the ends, and it is then centrifuged for 5 minutes at 2,500 r. p. m. The red cells are packed to one end, and resting upon them the buffy layer of the less dense leukocytes may be seen. Readings of the total cellular volume of the blood are made directly in per cent.

### *Part I. Chemical Analysis.*

After trial of various methods proposed in the literature the following were found most suitable and were used throughout this investigation.

*Blood Solids.*—One drop of blood was caught on a small piece (2 sq. cm.) of dried blotting paper, which was at once placed in a wide-mouthed weighing bottle (the stopper being inserted at once to avoid evaporation) and weighed. The stopper was then tilted, the whole placed in an air bath at 80–90°C., and dried to constant weight.

The solids range from 21 to 24 per cent, two cases only being a trifle outside of these limits (see table). From this series the value of 20 per cent as given by Myers and Fine<sup>3</sup> seems too low.

*Total Nitrogen of Blood, Plasma, and Serum.*—1 cc. of each (uniformly delivered and washed from a standardized 1 cc. bulb pipette) was used in the regular Kjeldahl process. The titrations were done with 0.05 N solutions.

#### *Total Nitrogen.*

	Gm. per 100 cc.
Blood*.....	3.0–3.7
Plasma.....	1.1–1.4
Serum.....	1.2–1.4

\*The value of 3.0 gm. of nitrogen in 100 cc. of blood as given by Myers and Fine,<sup>3</sup> seems to be the low level for normals.

*Non-Protein Nitrogen.*—In place of using either methyl alcohol or trichloroacetic acid for the precipitation of the protein substances, we use an acid mercuric chloride solution. Although

<sup>2</sup> Made by Emil Greiner Co., New York.

<sup>3</sup> Myers, V. C., and Fine, M. S., *The Post-Graduate*, New York, 1914–1915.

bichloride of mercury has been used as a protein precipitant ever since Schenck<sup>4</sup> suggested it, it has never been used in connection with non-protein nitrogen determinations in blood. The reason for this is that Schenck's 0.8 per cent hydrochloric acid and 2 per cent bichloride solution gives almost invariably a very cloudy filtrate, containing nitrogen other than non-protein. On trying stronger solutions of both the acid and the mercuric chloride, it was found that 5 per cent hydrochloric acid and a 5 per cent solution of bichloride always gives a water-clear filtrate. The advantage of this precipitant is that a perfect separation of protein from non-protein substances is obtained with only one filtration, whereas in the method of Folin and Denis<sup>5</sup> with methyl alcohol and zinc chloride, as well as in Greenwald's<sup>6</sup> with trichloroacetic and kaolin, two filtrations must be made, one after treatment with each reagent.

Furthermore, the time required for complete precipitation is reduced to a few minutes.

*Method.*—5 cc. of blood are pipetted into a small Erlenmeyer flask. 5 cc. of water are added to luke the blood, then 10 cc. of 5 per cent HCl solution; the mixture is well shaken, and 10 cc. of 5 per cent mercuric chloride solution are added, making in all exactly 30 cc. This is mixed well, then centrifuged thoroughly. The clear supernatant liquid is filtered through a small, dry filter paper; of the filtrate 20 cc. are taken for a micro-Kjeldahl, using 5 cc. of concentrated sulfuric acid and about 1 gm. of potassium sulfate. (Boiling for  $\frac{1}{2}$  hour is sufficient because the excess mercury from the precipitating agent serves here as a very efficient catalyst.)

The ammonia is determined by aeration and Nesslerizing, or, as done in this series, by distilling over into 20 cc. of 0.025 N sulfuric acid, and titrating back with 0.025 N sodium hydroxide, using Congo red as indicator.

The values range from 30 to 45 mg. per 100 cc. of blood. Three cases only went a trifle higher (see Composite Table). All values above 50 mg. should be regarded as pathological.

The following is for comparison of values obtained by various workers.

<sup>4</sup> Schenck, F., *Arch. ges. Physiol.*, 1894, iv, 203.

<sup>5</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

<sup>6</sup> Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

*Non-Protein Nitrogen.*

	Mg. per 100 cc. of blood.
This series.....	30-45
Folin and Denis <sup>7</sup> .....	22-37
Greenwald <sup>6</sup> .....	30
Bang <sup>8</sup> .....	19-39
Taylor and Hulton <sup>9</sup> .....	25-28
McLean and Selling <sup>10</sup> .....	23-44
Myers and Fine <sup>3</sup> .....	25-30
Hohlweg <sup>11</sup> .....	40-61

*Urea Nitrogen.*—Marshall's<sup>12</sup> urease method was used for decomposing the urea into ammonium carbonate; Folin's<sup>5</sup> aeration and colorimetric method for determining the same. The Duboseq colorimeter was used in all cases.

Values obtained range from 15 to 25 mg. of urea nitrogen in 100 cc. of blood. Only two cases fell below 15 mg. and none exceeded 25 mg. (see Composite Table).

*Urea Nitrogen.*

	Mg. per 100 cc. of blood.
This series.....	15 -25
Folin and Denis <sup>7</sup> .....	12 -27
Schwartz and McGill <sup>13</sup> .....	10.8-25.2
Bang <sup>8</sup> .....	6 -20
Myers and Fine <sup>3</sup> .....	12 -15
McLean and Selling <sup>10</sup> .....	12 -27

*Ammonia Nitrogen.*—Using 5 cc. of blood, the ammonia was aerated and determined colorimetrically according to the method of Folin and Denis.<sup>5</sup> The values obtained range from 0.4 to 0.75 mg. in 100 cc. of blood (see Composite Table).

*Uric Acid.*—The colorimetric method of Folin and Denis<sup>14</sup> was used, with the modification suggested by Benedict.<sup>15</sup>

<sup>7</sup> Folin and Denis, *J. Biol. Chem.*, 1913, xiv, 29; 1914, xvii, 487.

<sup>8</sup> Bang, I., *Biochem. Z.*, 1915, lxxii, 104.

<sup>9</sup> Taylor, A. E., and Hulton, F., *J. Biol. Chem.*, 1915, xxii, 63.

<sup>10</sup> McLean, F. C., and Selling, L., *J. Biol. Chem.*, 1914, xix, 31.

<sup>11</sup> Hohlweg, H., *Med. klin. Woch.*, 1915, xi, 331.

<sup>12</sup> Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 487.

<sup>13</sup> Schwartz, H., and McGill, C., *Arch. Int. Med.*, 1916, xvii, 42.

<sup>14</sup> Folin and Denis, *J. Biol. Chem.*, 1912-13, xiii, 469.

<sup>15</sup> Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 629.

The results seem to show that normal values of uric acid range between 1.0 and 3.5 mg. in 100 cc. of blood. Two cases fell below 1 mg. (see Composite Table).

The following is for comparison of values obtained by various workers.

*Uric Acid.*

	Mg. per 100 cc. of blood.
This series.....	1.0-3.5
Folin and Denis <sup>7</sup> .....	0.7-3.7
Maase and Zondek <sup>16</sup> .....	2.5
Myers and Fine <sup>3</sup> .....	1.0-2.0

*Creatine and Creatinine.*—Folin's<sup>17</sup> method was used for determining both the creatine and the creatinine.

Most of the values obtained for preformed creatinine are 0.1 mg. or less in 100 cc. of blood. It was not deemed worth while to estimate below 0.1 mg., which value is therefore given in the table whenever the actual amount was found to be this or less. Four cases were a trifle higher.

For creatine the values range between 3 and 6.5 mg. in 100 cc. of blood (see Composite Table).

*Creatine and Creatinine.*

	Mg. per 100 cc. of blood. Creatine.	Creatinine.
This series*.....	3.0- 6.5	0.1-0.5
Folin and Denis <sup>7</sup> .....	5.2- 8.1	1.1-1.4
Myers and Fine <sup>3</sup> .....	5.0-10.0	1.0-2.0

\*From this investigation it appears that creatinine is present normally in traces only and any value exceeding 1 mg. should be considered pathological.

*Residual Nitrogen.*—This value was obtained by subtracting from the total non-protein nitrogen the sum of the nitrogen values for urea, ammonia, uric acid, creatine, and creatinine. This undetermined nitrogen includes the amino-acid nitrogen. The values obtained range from 10 to 25 mg. in 100 cc. of blood. Only three cases fell below this value (see Composite Table).

<sup>16</sup> Maase, C., and Zondek, H., *Munch. med. Woch.*, 1915, lxii, 1110.

<sup>17</sup> Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

Bang<sup>8</sup> reports 3 to 22 mg. "amino-acid nitrogen" per 100 cc. of blood, but his figures indicate only the difference between total non-protein and urea nitrogen.

Van Slyke's<sup>18</sup> method for the quantitative determination of the amino-acids present was not used in this series, because it would necessitate the further withdrawal of more blood than we thought advisable.

*Blood Sugar.*—The original method of Lewis and Benedict<sup>19</sup> was chosen because of its accuracy and simplicity.

The values obtained range between 50 and 120 mg. per 100 cc. of blood (see Composite Table).

The following is for comparison of results obtained by various workers.

*Sugar.*

	Mg. per 100 cr. of blood.
This series.....	50-120
Lewis and Benedict <sup>19</sup> .....	90-110
Michaelis <sup>20</sup> .....	90-130
Strouse <sup>21</sup> .....	40-120
Taylor and Hulton <sup>9</sup> .....	50-150
Naunyn <sup>22</sup> .....	70-100
Liefmann and Stern <sup>23</sup> .....	70-110
Hollinger <sup>24</sup> .....	70-100
Bang <sup>25</sup> .....	100-110
Frank <sup>26</sup> .....	80-110
Rolly and Oppermann <sup>27</sup> .....	62- 88
Kowarsky <sup>28</sup> .....	50-110
Freund and Marchand <sup>29</sup> .....	55-120

<sup>18</sup> Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

<sup>19</sup> Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

<sup>20</sup> Michaelis, L., *Biochem. Z.*, 1914, lix, 166.

<sup>21</sup> Strouse, S., *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 211.

<sup>22</sup> Naunyn, B., *Diabetes Mellitus*, Vienna, 2nd edition, 1906.

<sup>23</sup> Liefmann, E., and Stern, R., *Biochem. Z.*, 1906, i, 299.

<sup>24</sup> Hollinger, A., *Deutsch. Arch. klin. Med.*, 1908, xcii, 217.

<sup>25</sup> Bang, *Biochem. Z.*, 1913, lvii, 300.

<sup>26</sup> Frank, E., *Z. physiol. Chem.*, 1910-11, lxx, 129.

<sup>27</sup> Rolly, F., and Oppermann, F., *Biochem. Z.*, 1912-13, xlvi, 187.

<sup>28</sup> Kowarsky, A., *Deutsch. med. Woch.*, 1913, xxxix, 1635.

<sup>29</sup> Freund, H., and Marchand, F., *Deutsch. Arch. klin. Med.*, 1913, cx, 120.

*Fat and Cholesterol.*—The methods of Kumagawa and Suto,<sup>30</sup> Rosenthal and Trowbridge,<sup>31</sup> and Bloor<sup>32</sup> are very time-consuming. We have therefore modified the Soxhlet method for fat in milk, so that it may be applied to blood. The ordinary method necessitates drying the material on a paper coil at 90–95°C. This cannot be done with blood, because of the ease with which the unsaturated fatty substances in blood are oxidized in air. In order to avoid oxidation, we consequently dry the blood in vacuum over  $P_2O_5$ . Furthermore, great stress must be laid on the use of anhydrous ether for the Soxhlet, otherwise foreign substances will be extracted along with the fats and cholesterol.

*Method.*—5 cc. of blood are allowed to run slowly from a pipette onto a very loose coil of fat-free absorbent paper; this is dried over night in a vacuum desiccator over  $P_2O_5$  at 50–60°C. The coil is then extracted with anhydrous ether in a Soxhlet for 5 hours. The ether is evaporated from the extracted material, leaving a residue not in the least contaminated by pigments. It is dried to constant weight *in vacuo* over  $P_2O_5$ . The residue thus obtained consists of fatty substances plus cholesterol. To determine the cholesterol, it is taken up with 5 cc. of chloroform, to which are added 2 cc. of acetic anhydride and 0.1 cc. of  $H_2SO_4$ , as directed by Autenrieth and Funk,<sup>33</sup> and also by Bloor.<sup>34</sup> The solution is made up to 10 cc. with chloroform, allowed to stand in the dark for 15 minutes, and then compared in a Duboscq colorimeter with a similarly treated standard solution of cholesterol in chloroform (5 mg. of cholesterol in 10 cc. are a good strength for comparison).

Values obtained for cholesterol range from 30 to 60 mg. in 100 cc. of blood (see Composite Table).

Subtracting the value of the cholesterol from that of the total ether extract gives the amount of fat plus fatty acids present (also lipoids present in traces). Values for fat plus fatty acids range from 60 to 160 mg. per 100 cc. of blood. One case was as high as 320 mg. (see Composite Table).

This method has the following advantages over those pre-

<sup>30</sup> Kumagawa, M., and Suto, K., *Biochem. Z.*, 1908, viii, 212.

<sup>31</sup> Rosenthal, H., and Trowbridge, P. F., *J. Biol. Chem.*, 1915, xx, 711.

<sup>32</sup> Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377.

<sup>33</sup> Autenrieth, W., and Funk, A., *Munch. med. Woch.*, 1913, lx, 1243.

<sup>34</sup> Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

viously in use: (1) Both fats and cholesterol may be determined in the same 5 cc. portion of blood. (2) It does away with the repeated ether extraction and washing in separatory funnels. (3) The actual time of manipulation is only 30 minutes, the drying and extraction needing no attention.

*Chlorides.*—Determinations were made both upon blood and plasma, using the method of McLean and Van Slyke.<sup>35</sup>

450 to 500 mg. of chlorides in terms of NaCl were obtained in 100 cc. of blood, and 560 to 640 mg. in 100 cc. of plasma (only four cases fell a little below these values; see Composite Table).

Previously reported results by other workers agree well with these averages.

*Total Chlorides as NaCl.*

	Mg. per 100 cc.	
	Blood.	Plasma.
This series.....	450-500	560-640
McLean and Van Slyke <sup>35</sup> .....	490	597-614
Rogée and Fritsch <sup>36</sup> .....	450	
Myers and Fine <sup>3</sup> .....		650

*Alkaline Reserve of Blood.*—Use was made of Van Slyke's<sup>37</sup> method for determining the carbon dioxide capacity of plasma at alveolar tension. From the burette readings the volume percentage of carbon dioxide bound as carbonate by the plasma was obtained by reference to Van Slyke's table. In his opinion, normals show 53 to 77 volume per cent of CO<sub>2</sub> chemically bound by plasma, figures lower than 50 per cent in adults indicating acidosis. All values obtained in this series agree with the above. The majority lie between 58 and 68 volume per cent, and none is below 55 (see Composite Table).

*Part II. Physical Analysis.*

*Conductivity of the Circulating Blood.*—A cell was devised<sup>2</sup> for determining the conductivity of the blood as it exists in circulation. It consists of three pieces: a center piece B, which is a tube 1½ inches long and ½ inch outside diameter, and having a capacity

<sup>35</sup> McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

<sup>36</sup> Rogée, H., and Fritsch, C., *Biochem. Z.*, 1913, liv, 53.

<sup>37</sup> Van Slyke, D. D., *Proc. Soc. Exp. Biol. and Med.*, 1915, xii, 7.





## SOME OBSERVATIONS ON THE TETANY OF PARATHYROIDECTOMIZED DOGS.

By ISIDOR GREENWALD.

(From the Harriman Research Laboratory, Roosevelt Hospital, New York.)

(Received for publication, April 7, 1916.)

In a previous communication, the author<sup>1</sup> called attention to the marked retention of phosphorus after parathyroidectomy in dogs. Later experiments<sup>2</sup> showed that this retention was followed or accompanied, but never preceded, by a retention of sodium and potassium. The amount of phosphorus in the blood and serum was found to be increased and this increase was chiefly, if not entirely, in those compounds of phosphorus which are insoluble in the usual lipid solvents but which are soluble in a mixture of dilute acetic, or hydrochloric, and picric acids. As this retention of phosphorus appears to be the most marked change in metabolism as yet observed after parathyroidectomy, it seemed desirable to investigate its significance further.

From the experiments of Gamgee, Priestly, and Larmuth<sup>3</sup> it is quite evident that if the phosphorus were retained as *o*-phosphate it could not be regarded as the cause of tetany. Experiments in this laboratory<sup>4</sup> confirmed the results of Gamgee, Priestly, and Larmuth as to the non-toxicity of relatively large amounts of sodium phosphate. It was found possible to increase the amount of acid-soluble phosphorus to twenty times the normal amount without marked effects on the behavior of the animals. Other phosphorus compounds were then sought as the possible toxic agents. Attention was immediately directed to inosinic acid. This substance, which is present in muscle tissue in con-

<sup>1</sup> Greenwald, I., *Am. J. Physiol.*, 1911, xxviii, 103.

<sup>2</sup> Greenwald, J. *Biol. Chem.*, 1913, xiv, 363, 369.

<sup>3</sup> Gamgee, A., Priestly, J., and Larmuth, L., *J. Anat. and Physiol.*, 1877, xi, 255.

<sup>4</sup> Greenwald, J. *Pharm. and Exp. Therap.*, 1915, vii, 57.

siderable quantity is, chemically, one of the simplest of the nucleic acids. The large amount present in a tissue so poor in nuclei as is muscle, and its ready extraction therefrom, indicate that, physiologically, it is something quite different. Besides phosphoric acid, it yields upon hydrolysis, a pentose, *d*-ribose, and hypoxanthine. The latter, according to Schmiedeberg,<sup>5</sup> produces convulsions in frogs. Moreover, the author had observed the effects of intravenous injections of xanthine in dogs and had been struck by their resemblance to the tetany of parathyroidectomized dogs. It was therefore determined to investigate the possible rôle of inosinic acid in the production of tetany.

Barium inosinate was prepared by the method of Haiser and Wenzel.<sup>6</sup> It was found that when added to horse serum, both the phosphorus and the hypoxanthine could be quantitatively recovered from the filtrate obtained by treating the serum with a saturated solution of picric acid in 1 per cent acetic acid. The phosphorus was determined by the method of Pouget and Chouchak as adapted by the author<sup>7</sup> to the estimation of phosphorus in serum. For the determination of the purine nitrogen, larger amounts of the picric acid filtrate were evaporated on the water bath to a small volume. The entire mass, including the crystallized picric acid, was transferred to an apparatus for the continuous extraction of liquids and there treated with benzene until no more picric acid was removed. About 5 per cent, by volume, of concentrated sulfuric acid was then added and extraction continued. After this was apparently complete the aqueous solution was heated to drive off the residual benzene, cooled, made alkaline with ammonium hydroxide, and treated with ammoniacal silver solution. No precipitate was obtained from the equivalent of 225 cc. of normal horse serum, but if barium inosinate had been added a heavy white precipitate appeared. This was filtered off, washed with dilute ammonium hydroxide, and decomposed with hydrochloric acid. The silver chloride was filtered out, the filtrate neutralized with sodium hydroxide, made acid with acetic acid, and the hypoxanthine reprecipitated in the usual manner.

<sup>5</sup> Schmiedeberg, O., *Ber. chem. Ges.*, 1901, xxxiv, 2552.

<sup>6</sup> Haiser, F., and Wenzel, F., cited by Steudel, H., *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1910, ii, 602.

<sup>7</sup> Greenwald, J. *Biol. Chem.*, 1915, xxi, 29.

with copper sulfate and sodium hisulfite. The preeipitate was filtered out, washed with hot water, and its nitrogen content determined. The amount found corresponded with the amount of barium inosinate added.

When this method was applied to dog serum it was found that neither normal serum nor that of parathyroidectomized dogs in which tetany was just developing contained any purine nitrogen. In two experiments, in which the serum was not absolutely clear, a trace of purine nitrogen was obtained, but this may have been due to the nuclear material of the cells.

In order to test further the possibility of inosinic acid being the toxic agent in tetany, a solution of sodium inosinate, prepared from the barium salt and sodium sulfate, was injected intravenously into a dog from which the thyroid and parathyroids had been removed 5 hours previously. No effect was observed, although the amount used was greater than could have been tolerated if inosinic acid were the toxic agent in tetany. Determinations made after the injection of the sodium inosinate and also when tetany appeared on the following day showed that at the latter time the amount of acid-soluble phosphorus in the serum was less than in the former instance.

The hypothesis that inosinic acid is the toxic agent in the causation of the tetany of parathyroidectomized dogs must therefore be abandoned and the significance of the retention of phosphorus remains, for the present, unexplained.

*Recovery of the Phosphorus of Barium Inosinate Added to Serum.*

	mg.	mg.	mg.
Acid-soluble phosphorus in 1 cc. of serum.	0.063	0.061	0.018
Phosphorus in added barium inosinate....	0.058	0.049	0.013
Total.....	0.121	0.110	0.031
Found.....	0.117	0.116	0.031

*Recovery of the Nitrogen of Barium Inosinate Added to Serum.*

	mg.	mg.
Nitrogen of the barium inosinate added to 250 cc. of serum .....	22.09	24.07
Purine nitrogen found.....	19.45	23.47

## 226 Tetany of Parathyroidectomized Dogs

### *Experiment Showing the Effect of the Intravenous Injection of Sodium Inosinate.*

To a solution of 1.8345 gm. of barium inosinate in 100 cc. of hot water there was added 0.460 gm. of dry sodium sulfate dissolved in water. After standing over night, the mixture was filtered and the filtrate and washings were diluted to 150 cc. The solution contained no barium and only a trace of sulfate. The liquid was then made approximately isotonic with blood by the addition of 0.60 gm. sodium chloride.

Dec. 11, 1914. Dog weighing 4.18 kilos. Parathyroidectomy at 11 a.m. Between 4.00 and 4.27 p.m., injected 145 cc. of the above solution into a femoral vein, using cocaine anesthesia. No symptoms. 4.43 p.m., drew a small sample of blood. Serum obtained contained 0.127 mg. acid-soluble phosphorus in 1 cc.

Dec. 12, 5 p.m. Fairly well marked twitching. Bled to death under cocaine anesthesia. The serum obtained from the first few cc. of blood contained 0.097 mg. acid-soluble phosphorus in 1 cc.

# THE FORMATION OF SPECIFIC PROTEOCLASTIC FERMENTS IN RESPONSE TO INTRODUCTION OF PLACENTA.

By FLORENCE HULTON.

(From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia.)

(Received for publication, May 4, 1916.)

The digestive power on pure proteins of the serum of injected rabbits was reported in a previous paper.<sup>1</sup> These results showed plainly "that under the conditions of the experiment there is practically no digestion with the blood of the injected animal in excess of that which takes place with the serum of the normal control animal." There were two other points that it seemed wise to investigate: (1) the digestive action on placental protein of the serum of an animal injected with placenta; (2) the digestive action of this same serum on various pure proteins.

Two dogs were given three injections of a placental emulsion, a week apart. The placenta used was human placenta, procured soon after delivery. All fetal remnants were carefully dissected away from the placenta, immediately after delivery with sterile instruments, and the upper and lower surfaces cut off. It was then wrapped in sterile gauze and placed in a desiccator over chloroform. As soon as it was received in the laboratory, it was ground in a sterile grinder, caught in a sterile dish, extracted with Ringer's solution, and strained through sterile gauze. The first two injections were 10 cc. each, the first given intraperitoneally, the second intramuscularly. The third injection of 20 cc. was administered 10 cc. intraperitoneally, and 10 cc. intramuscularly. The following day the dogs were etherized and bled to death from the carotid, the blood being caught in a beaker and stirred constantly to defibrinate. Two normal dogs were bled in the same manner.

<sup>1</sup> Hulton, F., *J. Biol. Chem.*, 1916, xxv, 163.

*Experiment Showing the Effect of the Intravenous Injection of Sodium Inosinate.*

To a solution of 1.8345 gm. of barium inosinate in 100 cc. of hot water there was added 0.460 gm. of dry sodium sulfate dissolved in water. After standing over night, the mixture was filtered and the filtrate and washings were diluted to 150 cc. The solution contained no barium and only a trace of sulfate. The liquid was then made approximately isotonic with blood by the addition of 0.60 gm. sodium chloride.

Dec. 11, 1914. Dog weighing 4.18 kilos. Parathyroidectomy at 11 a.m. Between 4.00 and 4.27 p.m., injected 145 cc. of the above solution into a femoral vein, using cocaine anesthesia. No symptoms. 4.43 p.m., drew a small sample of blood. Serum obtained contained 0.127 mg. acid-soluble phosphorus in 1 cc.

Dec. 12, 5 p.m. Fairly well marked twitching. Bled to death under cocaine anesthesia. The serum obtained from the first few cc. of blood contained 0.097 mg. acid-soluble phosphorus in 1 cc.

TABLE II.  
*Increase of Non-Protein N in 100 Cc. of Serum.*

Digestion with	Injected animals.*	Normal animals.
	mg.	mg.
Placental protein.....	19	41
•	28	39
Protamine.....	223	169
Casein.....	58	121
Bence-Jones protein.....	33	51
Phascolin.....	55	50
Edestin.....	25	31
Gliadin.....	125	42
Soy bean globulin.....	26	30
Milk albumin.....	25	22

\* Injected animals in each case were injected with human placenta emulsion.

The results show that, under the conditions of the experiment, with placental protein there is no increase in the digestive powers of the serum of the injected animal, that of the normal animal showing a slightly greater rise in non-protein N. The greatest amount of digestion with pure proteins has taken place with protamine. This might be explained on the ground that protamine, being one of the simpler proteins, is more readily split into its amino-acid components. Gliadin is split to a marked degree by the blood of the injected animal.

The figures indicate that the blood of the dog possesses to a marked degree proteoclastic activity, which is not increased toward either placental protein or the other proteins used in the experiment by injecting placental proteins. This activity may be due to ferments present as normal constituents of the blood or appearing there under certain metabolic conditions, just as trypsin appears in the urine. Any specific reaction in pregnancy or in neoplastic conditions it would seem could hardly be due to increased ferment production in the blood and the results do not bear out the statement that placental proteins in the circulating blood call forth a protective enzyme which by digestion removes the placenta from the blood.



## SUMMARY.

Placental protein is not digested to any greater degree by the serum of an animal sensitized to placenta than by the normal serum.

The digestive power of the serum of an animal sensitized to placenta is not increased for casein, Bence-Jones protein, phaseolin, edestin, soy bean globulin, or milk albumin. Casein is digested to a marked degree by the normal serum, and in most cases the normal serum possesses the more marked activity.

Protamine is digested to a marked degree in both cases, the injected animal showing increased activity.

Gliadin is not digested to any great extent by the normal serum, but is by the serum of the injected animal.

In general it may be said that the injection of placenta does not increase the general or call forth the specific proteoclastic ferment of the blood.

I take this opportunity to thank Dr. A. E. Taylor, under whom the work was begun, for his interest and help, and also Dr. M. M. Peet, of the Department of Research Surgery, for his help in bleeding the normal dogs.

# A COLORIMETRIC METHOD FOR THE ESTIMATION OF FREE FORMALDEHYDE AND HEXAMETHYLENAMINE.

By R. J. COLLINS AND P. J. HANZLIK.

(From the Pharmacological Laboratory, Western Reserve University, Cleveland.)

(Received for publication, April 24, 1916.)

It has previously been ascertained<sup>1</sup> that the phloroglucinol or Jorissen test is among the most sensitive color tests for free formaldehyde. The advantages claimed for it are: (1) simplicity; (2) great sensitivity; (3) it reacts with free or liberated formaldehyde only, and does not itself liberate formaldehyde from such compounds as hexamethylenamine; (4) finally, with the phloroglucinol test there is a gradation of colors, from pink to deep red, depending on the concentration of formaldehyde, which suggested to us the possibility of its application in a quantitative manner. However, the colors are not permanent. Their maximum intensity is reached within 3 minutes and then they gradually change to a violet and finally disappear altogether. This is particularly true with the high dilutions. Therefore we sought for a substance, or mixture of substances, which would give us the same quality of color as in the phloroglucinol test, and yet be permanent when used in making a set of standards.

After considerable experimentation with metallic salts and different dyes, a mixture of Congo red (0.025 per cent in water containing 5 per cent alcohol) and methyl orange (0.01 per cent in water) was found to be satisfactory. A mixture of these in the proper proportions is only necessary in matching the reds of phloroglucinol in the lower concentrations of formaldehyde. For the higher concentrations Congo red alone suffices.

<sup>1</sup> Hanzlik, P. J., and Collins, R. J., *Arch. Int. Med.*, 1913, xii, 578. The reagent consists of phloroglucinol (reagent-Merck) 0.1 gm. dissolved in 10 cc. of 10 per cent sodium hydroxide.

It is important to note that samples of Congo red, even of the same manufacture, are apt to differ considerably in point of quantity, but apparently not in quality, of color. Owing to this it is necessary to standardize any particular specimen of Congo against a known standard made from an inorganic salt before it can be used to prepare the permanent Congo standard for the dilutions. We believe that we have successfully achieved this with potassium bichromate and sulfuric acid. The following procedure for standardizing Congos has been found to work satisfactorily.

1.7616 gm. of  $K_2Cr_2O_7$  by titration against alkali (roughly about 30 cc. of a 5 per cent solution) and 11.5537 gm. of absolute  $H_2SO_4$  (about 7 cc. concentrated) are mixed and diluted to the mark in a 50 cc. Nessler tube with a column of 12 cm. This is equivalent to 50 cc. of 1:100,000 absolute formaldehyde, or 50 cc. of a Congo standard 1:100,000, which contains 0.000625 gm. of the original dry Congo red, or 2.5 cc. of 0.025 per cent. It is only necessary, therefore, to prepare a proper mixture of  $K_2Cr_2O_7$  and  $H_2SO_4$ , and when an unknown Congo solution is standardized against this, the quantity used will contain 0.000625 gm. of Congo. The strong solution can then be diluted or made accordingly, the standard dilutions from this to be equivalent to the different concentrations of formaldehyde.

The proportions of Congo red and methyl orange which have been worked out for the different concentrations of absolute formaldehyde are presented in Table I. The basic solutions for making the standards are not mixed until ready for use. We have kept both solutions for 2 to 3 months without any demonstrable change. However, after the dyes are mixed and diluted to the proper volume with water, deterioration occurs with the weaker colors within a week, the stronger colors remaining permanent for at least 2 weeks. It is advisable, therefore, to prepare a set of dilutions each day. The technique of making standards consists simply in measuring the quantity of each solution necessary with an accurately graduated pipette into 50 cc. Nessler tubes of the short variety with columns of fluid 12 cm. high. These are then diluted with water to the mark, gently agitated, and are ready for use.

For the estimation of formaldehyde in clear aqueous solutions

containing formaldehyde, the technique is as follows: An aliquot portion, 1, 5, or 10 cc., of the solution are measured into the Nessler tube, phloroglucinol reagent (1 to 2 cc.) is added, and the whole is diluted to the 50 cc. mark and gently agitated. After standing 3 minutes the tube is matched against a series of tubes containing the standard colors just described. The results can be expressed in percentage or gm. of formaldehyde. In calculation allowance must be made for dilution in the Nessler tube.

TABLE I.  
*Standard Mixtures of Congo Red and Methyl Orange.*

Concentration of formaldehydo.*	Percentage concentration of formaldehyde	Congo red** 0.025 per cent.	Methyl orange** 0.01 per cent
		cc.	cc
1: 20,000	0.005	20 0	0
1: 30,000	0.0033	11 0	0
1: 40,000	0.0025	9 0	0
1: 50,000	0.002	8 0	0
1: 60,000	0.0016	5 0	0
1: 80,000	0.00125	4 0	0
1: 100,000	0.0010	2 5	0
1: 200,000	0.0005	0 85	0 40
1: 250,000	0.0004	0 65	0 35
1: 500,000	0.0002	0 25	0 18
1: 750,000	0.00014	0 20	0 15
1: 1,000,000	0.00010	0 13	0 10

\* Total volume of solution = 50 cc. in a Nessler tube with a 12 cm column.

\*\* The quantities of Congo red and methyl orange here indicated are mixed and diluted with water to the mark in a 50 cc. Nessler tube with a 12 cm. column.

For instance, if 5 cc. of the formaldehyde solution were used and diluted to 50 cc., then the percentage concentration to which the matched color corresponds is multiplied by 10. This gives the percentage concentration of the formaldehyde solution. From this the absolute weight in gm. of formaldehyde can, of course, be easily calculated.

For urine the procedure must be slightly modified because of the presence of phosphates which, when precipitated by the alkali of the phloroglucinol reagent, interfere with the reading of

the color. These are removed by adding to an aliquot portion of the urine a few drops of concentrated (50 per cent) sodium hydroxide and filtering, then washing with a little water to the original volume.

If the urine is concentrated or deeply colored, an equal volume of the same or some other urine of about the same color must be added to the standards containing the mixtures of Congo red and methyl orange. The estimation is then carried out in exactly the same way as for aqueous solutions.

We have compared the colorimetric method with the Romijn or iodine method,<sup>2</sup> the U.S.P. peroxide method with heat,<sup>3</sup> and the sodium hydroxide heat pressure method.<sup>4</sup> Distillates from known quantities of hexamethylenamine were used. Results illustrative of the data obtained are indicated in Table II. The following mean percentage recoveries of the theoretical yield of formaldehyde were obtained with the different methods: colorimetric, 99 per cent; Romijn, 88 per cent; U.S.P., extremely variable (none to 96 per cent); hydroxide and pressure, extremely variable (none to 90 per cent). In order to be able to obtain these percentage recoveries with the other methods usually ten times the quantity of the distillate was necessary for single estimations, as compared with the colorimetric. It was also found that the colorimetric method is more accurate with higher dilutions of formaldehyde, because it is more difficult to read small differences between more intense than between weaker colors.

For direct application to urine the other methods with which the colorimetric was compared are not suitable, since urine itself consumes iodine, hydroxide, and peroxide. Distillation of urine containing such an easily decomposable formaldehyde compound as hexamethylenamine is not permissible if an idea of the formaldehyde liberated during its passage through the body is to be obtained. Here the only choice is the colorimetric method.

Salkowski<sup>5</sup> has recently practiced a modification of the Leach ferric chloride test as a colorimetric method for the estimation of formaldehyde.

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<sup>2</sup> Sutton, F., *Volumetric Analysis*, Philadelphia, 9th edition, 1904, 370.

<sup>3</sup> U. S. Pharm., 8th revision, 1900, 266.

<sup>4</sup> Smith, C. E., *Am. J. Pharm.*, 1898, lxx, 86.

<sup>5</sup> Salkowski, E., *Biochem. Z.*, 1915, lxxviii, 337; 1915, lxxi, 365.

In this hydrochloric acid is used, and this precludes its use for the estimation of free formaldehyde in the presence of easily decomposable formaldehyde compounds, such as hexamethylenamine. We have not practiced the phenylhydrazine-nitro-prusside test for quantitative purposes because of difficulties with the test which were pointed out in a previous paper. It has been used by Dunning.<sup>6</sup>

TABLE II.

*Estimation of Formaldehyde in Hexamethylenamine.*

Amount of hexamethyl- enamine.	Equivalent in HCHO.	Formaldehyde recovered.			
		Romijn.	U. S. P.	Alkali.	Colorimetric.
gm.	gm.	per cent	per cent	per cent	per cent
1.0	1.28	51(4)* 75(40)	None(4)		98(4)
0.5	0.64	53(7.8) 76(78)	32(7.8) 86(78)	41(7.8) 90(78)	100(7.8)
0.05	0.064	82(25) 96(100)	87(25) 96(100)	90(25)	96(8.2) 98(8)
0.005	0.0064	None(22) 96(220)	None(22) " (100)	None(22) " (100)	100(7.8) 100(22) 102(15)

Hexamethylenamine in each case above was decomposed with the aid of weak acid (three drops of 85 per cent phosphoric).

1.0	1.28	None(1) 78(10)	None(1)	None(1) 82(10)	78.1(1)
0.5	0.64	None(1) 74.25	None(1) 69(20) 93(25)	None(1) 78(20)	78.1(1)
0.05	0.064	74.7(25) 98(100)	76(100)		78.1(25)
0.005	0.0064	None(25) 48(50)	None(25)	None(25)	78.1(25)

Hexamethylenamine in each case was decomposed with the aid of strong acid (20 cc.), sometimes phosphoric, sometimes sulfuric.

\* The figures in brackets denote the number of cc. of distillate used for estimation. The distillate in each case measured 1,000 cc.

<sup>6</sup> Dunning, H. A. B., *J. Am. Pharm. Assn.*, 1914, iii, 637.



# FURTHER STUDIES ON THE NUTRITIVE DEFICIENCIES OF WHEAT AND GRAIN MIXTURES AND THE PATH- OLOGICAL CONDITIONS PRODUCED IN SWINE BY THEIR USE.\*

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(From the Departments of Agricultural Chemistry and Anatomy of the University of Wisconsin, Madison.)

PLATES 1 TO 5.

(Received for publication, April 8, 1916.)

In earlier studies on the influence of restricted natural feeds on growth and reproduction it was observed that a ration from the wheat plant<sup>1</sup>—wheat grain plus wheat straw—was wholly inadequate with heifers for reproduction and in some instances for continued growth. With swine, confined to the wheat grain and a suitable salt mixture, growth soon ceased and the animals passed into a poor condition, while a corn and salt mixture ration was at least sufficient for slow growth and continued well-being.<sup>2</sup> Similar results are on record with rats, and only when a liberal supply of casein and fat-soluble A was added to a wheat grain and salt mixture was growth continuous and, in the case of rats, reproduction possible.

Previous work in this laboratory has shown the necessity of two hitherto unappreciated factors in the diet of the growing animal. One of these is associated with butter fat and certain other fats and is found also in relatively small amounts in the grains, but in considerably higher concentration in the leaves of certain plants, as alfalfa and cabbage, than in the grains. The

\* Published with the permission of the Director of the Agricultural Experiment Station.

<sup>1</sup> Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Exp. Station, Research Bull.* 17, 1911.

<sup>2</sup> Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.



growth curves of two animals receiving Ration 1, consisting of 95.5 pounds of wheat meal, 2.5 pounds of wheat gluten, 2 pounds of butter fat, 323 gm.  $K_2HPO_4$ , and 513 gm. Ca lactate. The salt mixture is one which when added to a mixture of 70 parts of corn meal and 30 parts of gluten feed was adequate for a fair rate of growth and perfect maintenance. The protein content of the ration was approximately 13 per cent. Symptoms of malnutrition and failure to grow appeared only after a lapse of 9 months, and then loss of weight set in, followed by difficulty in locomotion, rough coat, labored breathing, and muscular twitching. Fig. 1 shows the appearance of one of these animals before the last stages. At this time this animal could still walk, although with difficulty, but there was not the extreme prostration appearing in other animals on similar diets and manifested by walking on the knees or dragging the rear quarters.

The animal was killed and the suprarenals, thyroid, parathyroid, spinal cord, and brain were removed. There were no certain abnormal conditions noted in the ductless glands. The spinal cord, which was examined by Dr. Miller, did show marked histological changes and the studies recorded for this animal, as well as others described below, are confined to the spinal cord. The vertebral canal contained a considerable quantity of fluid and the motor cells of the spinal cord exhibited marked alterations. Fig. 3 is a microphotograph of this pathological cord from the lumbar region. Fig. 2 shows a section of a normal pig's spinal cord in the same region and taken from an animal of approximately the same age as that of the pathological specimen. It will be noticed that there are marked differences in these specimens, although both were carried simultaneously through the same fixing and staining baths.<sup>9</sup>

In the normal cord the motor cells are not compressed and shrunken and are not surrounded by any appreciable quantity of fluid. The processes are intact, the nucleus and nucleolus remain distinct, and the Nissl granules likewise are distinct and separate. In the pathological cord we see a picture of marked contrast. The motor cells, surrounded by fluid, are compressed, the processes partly degenerated, the nucleus is shrunken, and the whole cell content stained as a uniform blue mass (black in photo).

<sup>9</sup> Blocks about 1 cm. thick were taken from the various regions of the spinal cord and throughout the series were treated as follows:

1. Fixed in Van Gehuchten's fluid for 8 hours.
2. Treated with alcohol of gradually increasing strength.
3. Cleared in a mixture of 4 parts of cedar oil and turpentine.
4. Embedded in paraffin.
5. Sections cut 5 and 7  $\mu$ .
6. Stained with erythrochrome.
7. Mounted in balsam.

These pictures of the cord changes are similar to those described by Vedder and Clark<sup>10</sup> for polyneuritis in fowls, but here produced in the presence of all known essentials for adequate nutrition. It would appear, therefore, that the inherent toxicity of the wheat kernel must be held directly responsible for these changes. The only possible criticism that could be raised against this conclusion is the factor of the quantity of fat-soluble A introduced into the diet. While 2 per cent of fat-soluble A (2 per cent of butter fat) is inadequate for normal growth of rats on a purified food diet, yet there is no evidence that this amount would not allow slow growth and continued maintenance of well-being in the animal. Further, in the diet here used there was undoubtedly some fat-soluble A introduced with the whole wheat grain.<sup>11</sup> The quantity of water-soluble B introduced was also probably ample, since the embryo of the wheat grain constituted about 5 per cent of this ration and that proportion is known to carry enough of this material for continued and normal growth.<sup>7</sup>

The pathological changes in the cord correlate very readily with some of the external symptoms manifested by these animals, such as stiffness and lack of muscular coordination.

*Experiment 2.*—In Text-fig. 2 is shown the growth curve of two female animals receiving 3 pounds of whole milk daily per individual, and what wheat meal they would consume (Ration 2). The wheat meal varied with the age of the animals from 2 to 4 pounds per individual daily, and constituted about 90 per cent of the dry matter of the ration. The plane of protein intake was between 12 and 15 per cent, and the butter fat consumed daily approximated 45 gm. This would make the per cent of butter fat in the ration vary from 2 to 4 per cent, depending upon the variation in the wheat meal consumed with increasing age. The milk protein constituted from 20 to 40 per cent of the total protein intake, likewise depending upon whether the animal was consuming 2 or 4 pounds of the wheat meal.

Growth was rapid and normal for 4 to 5 months, at which time the rate of growth began to decline and finally actual loss of weight set in. At this time there was extreme stiffness, twitching of the muscles, and dragging of the rear quarters. After being helped to stand these animals would fall again to their knees, draw the hind legs toward the fore feet, and from this position roll to one side. Fig. 4 shows these individuals. Postmortem examination was made by Dr. Miller and revealed the probable causes of the condition. The suprarenals, thyroid, parathyroid, and brain were re-

<sup>10</sup> Vedder, E. B., and Clark, E., *Philippine J. Sc.*, B, 1912, vii, 423.

<sup>11</sup> McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 179.

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moved. Grossly, the ductless glands showed no marked change. The brain was not sectioned. The spinal canal contained a considerable quantity of fluid and in the spinal cord again distinct changes had occurred (see Fig. 5). The motor cells were shrunk, surrounded by fluid, and appeared to be under pressure. The processes had degenerated, the Nissl granules were crowded together, and the nucleus and nucleolus nearly obliterated. It should be noticed in the microphotograph that some of the motor cells were apparently intact and not all of them in the same degree of degeneration. The histological picture here presented is very similar to that described for the animals on Ration 1. The ventrolateral and the ventromedial groups of cells in the anterior horn of the spinal column are chiefly affected. In other words, those cells that are most concerned in the formation of the motor root of the spinal cord showed greatest degeneration.

Apparently swine were somewhat more susceptible than rats to the toxic substance of wheat, and it may well be suspected that other species will vary in this respect. Unpublished data indicate that chickens are considerably less susceptible to a restricted wheat ration than any of the animals so far involved in these inquiries. It should again be emphasized that all the necessary factors for continued growth were present in this ration and the only explanation of these results must lie in the presence of toxicity. It was, however, possible that in Experiment 2 described above there was an inadequate supply of salts. The introduction of but 3 pounds of milk daily may have given an insufficient quantity of this factor, especially after the animals reached the higher weights of 150 pounds or more.

*Experiment 3.*—For the reason stated above, animals were started on Ration 3, composed of 95.5 pounds of wheat, 2.5 pounds of wheat gluten, 323 gm.  $K_2HPO_4$ , 513 gm. Ca lactate, and 3 pounds of whole milk per individual daily. The salt mixture is the same one used with success in experiments with corn grain. For 6 months growth was practically normal (see Text-fig. 3) but after that time decline set in and the animals suffered in nutrition as is indicated in Fig. 6. This figure shows one of the individuals toward the end of the experiment.

The gross external symptoms were similar to those previously described for other animals on excessive amounts of whole wheat. No microscopical examination of the cord of these animals was made, but we have no doubt that the picture would have been similar to that already described.

It is apparent from the results of the above experiments that too large a proportion of whole wheat in the ration will introduce

such a mass of toxic material as to render it impossible for this animal to complete its cycle of life, even in the presence of a considerable quantity of all known factors necessary for growth.

The question of the cause of this pathological condition may, however, be more complex than would seem evident from the above experiments. In those experiments described it is fairly clear that the result is due to the introduction of toxic material. In two additional experiments which we wish to present, the factors operative may be more complex and raise the question whether there are not several ways by which this pathological condition may be produced. In 1914 we published<sup>2</sup> among other data the curves of growth of swine receiving a ration of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, and 10 parts of oil meal, 323 gm. of  $K_2HPO_4$ , and 513 gm. of Ca lactate. With this ration, presumably carrying a suitable salt mixture, a fair quality of protein, but probably a rather low quantity of fat-soluble A and the toxicity of the wheat embryo of the middlings, growth was not sustained and the animals passed into a pathological condition. The factors operative in this experiment may have been toxicity augmented by too low a supply of fat-soluble A. Of course, the mass of toxicity in this ration was appreciably lower than in a ration carrying 90 per cent or more of whole wheat. The embryo of the wheat grain constitutes about 5 per cent of the grain, which would mean a content of 4 to 5 pounds of embryo in 100 pounds of the wheat ration described in Experiments 1, 2, and 3. In the mixed grain ration the content of embryo would be much less, probably not over 2 pounds in 100 pounds of the mixture, based on the fact that wheat middlings contain about 6 per cent of this material.

*Experiment 4.*—In this experiment the ration (Ration 4) consisted of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, 10 parts of oil meal, and a small daily allowance of roots and alfalfa. The latter did not constitute over 5 per cent of the dry matter of the ration. In this ration toxicity of the embryo was introduced and at the same time the salt content of the ration was inadequate and probably the content of fat-soluble A low. Text-fig. 4 shows the curves of growth and Fig. 8 the condition of the animals at the time the experiment was discontinued. After 4 to 5 months growth had ceased, although at no time was it normal. The animals gradually passed into a poor condition, accompanied by lack of muscular coor-

dination, emaciation, and labored respiration. They were also unable to stand.

One was killed and the spinal cord was examined by Dr. Miller. Fig. 7 shows a section of the cord in the cervical region. Besides a considerable collection of fluid in the vertebral canal the motor cells were surrounded by fluid, compressed, the processes degenerated, and the general condition of the cord similar in kind, if not in degree, to those already described. In this cord there was a smaller number of motor cells in a degenerated condition than in some of the cords examined, but sufficient to connect directly the physical state of the animal with the condition found in the nervous tissue. It should be noted that in some cases the stainable material of the cell has collected on one side and in mass. The tigroid bodies are no longer discrete.

*Experiment 5.*—Since in all the experiments so far described wheat has either constituted a large percentage of the ration, or else its by-product, such as middlings, carrying the embryo, has made up a considerable fraction, we are led to the conclusion that the pathological condition of these animals resulted from toxicity inherent in the wheat. We believe that this is the correct explanation in these cases. However, evidence is accumulating from our experiments with swine that this pathological condition may probably be produced by rations free from any known toxicity and abundantly supplied with fat-soluble A and water-soluble B, but inadequate only in the quality and quantity of its mineral content. On a ration consisting of 45 parts of corn meal, 45 parts of oats, 10 parts of oil meal, and 5 parts of butter fat, the animal will, after a sufficient lapse of time, cease to grow and pass into a condition shown in Fig. 9 (Text-fig. 5 shows the curves of growth). No histological examination has yet been made of the cord of any of the animals of this group, and we withhold final conclusions until more data are available; but the indications are rather positive toward the possibility of at least gross disturbances arising similar to those already described and involving the animal in external symptoms identical with those induced by the inherent toxicity of the wheat grain. In this ration the salt mixture is that common to the grains used. Unless later investigation should disclose toxicity in some of the products used, this would seem to be the logical explanation of our results.

Up to the present time we have found alfalfa meal or meat scraps among natural foodstuffs to be very successful supplements to a ration made up of corn, oats, middlings, and oil meal. There are undoubtedly many other materials that are either successfully in use or will be found capable of acting as supplements, but it is clear that if it is a single substance it must carry supplementing proteins, abundance of fat-soluble A, and especially a better salt mixture. We have no doubt that milk would readily come into this category if used in sufficient quantities to furnish an

adequate salt mixture. We have, however, records where skim milk was used in quantities sufficient to constitute as dry matter 1 to 5 per cent respectively of the dry matter of the ration. In these cases the grain ration was again made up of corn, oats, middlings, and oil meal. The protein level was approximately the same as in all the other experiments, 12 to 15 per cent. The results were a failure, as shown in the growth curves (Text-fig. 6) for the high intake of milk used. Failure was probably due to too low an intake of fat-soluble A and an inadequate supply of salts.

Where alfalfa constituted 20 to 25 per cent of the ration and middlings 20 per cent, the toxicity of the middlings was overcome, the inadequacy of the grains disappeared, and the animals remained sound and vigorous. Undoubtedly other roughages as the leaves or stems of plants would serve similar purposes. Curves of growth of these animals are shown in Text-fig. 7. This growth is somewhat below normal, possibly due to an absence of a highly efficient protein mixture.

As previously stated, it does not appear possible to supplement successfully the mixed grain ration here used with a single essential factor of growth. We have, however, in earlier experiments shown that the corn grain can be supplemented with salts alone with success in growth and maintenance, but here too the growth is somewhat below normal, due to poor proteins. With our mixed grain ration salts were used in the presence of middlings and a failure resulted. Butter fat has been imposed on the same ration and also on a mixture of corn, oats, and oil meal, with failure. It is probable, therefore, that our success with alfalfa rests on the introduction of a number of factors, such as more fat-soluble A and a better salt mixture. Whether the latter quality lies partly in its high calcium content remains for further study. These results with alfalfa make it clear how successful growth, even with omnivora, can be attained on a strictly vegetable diet,<sup>12</sup> and what an important part the leaf and stem portions of plants play in the life of herbivora.

With commercial meat scraps as supplements to a corn, middlings, oats, oil mixture as previously described, we have also had

<sup>12</sup> Hart and McCollum, *Proc. Am. Soc. Animal Production*, 1915; *J. Biol. Chem.*, 1916, xxiv, p. xxviii.

splendid success in obtaining normal growth and sustained vigor. This is a commercial product obtained from the packing establishments and carries considerable quantities of bone material. The calcium content of the material was 6.5 per cent. This product had been heated to 110°C., but the length of time could not be definitely given.

Curves of growth with both 1 per cent of meat scraps in the ration and with 5 per cent are shown in Text-figs. 8 and 9. These animals remained in splendid form and on 5 per cent have given birth to two litters of young. The reproduction, however, is not entirely satisfactory, a small percentage of the young being dead. Those that lived were vigorous and strong. On 1 per cent of meat scraps growth was splendid and maintenance perfect, but no successful reproduction has followed. The animals were physically active, conceived, but the young were carried from 1 to 2 weeks over time and all were born dead and hairless. On both diets carrying meat scraps the mothers have reached weights of 400 pounds or more. The factors introduced by this material were, again, better proteins, more fat-soluble A,<sup>13</sup> and an appreciable increase in calcium and phosphorus through the content of bone material in the meat scraps. It is indeed surprising to find as low an amount as 1 per cent of meat scraps supplementing these grains perfectly for growth, although apparently inadequate for successful reproduction. Our contention that alfalfa and meat scraps supplement the grains by virtue of introducing into the ration a better salt mixture, more fat-soluble A, and in the case of meat better proteins, is not wholly based on indirect inference, but on direct experimental inquiry. When a ration of corn, middlings, oats, and oil meal mixture has been supplemented with known salt additions and butter fat, thereby improving the mineral content of the ration and increasing the quantity of fat-soluble A, the curves of growth approach the normal and the animals appear at the present writing in normal condition. They have been on this ration for 100 days.

<sup>13</sup> Osborne and Mendel (*J. Biol. Chem.*, 1915, xx, 379) have shown the occurrence of fat-soluble A in beef fats

## SUMMARY.

Malnutrition, histologically characterized by nerve degeneration, may result from the absence of certain factors in the diet as in the case of beri-beri. A similar condition may likewise arise from the presence of toxic materials in apparently normal food products, and in the presence of all known factors essential for continued growth and well-being.

1. It is apparent from the results here recorded that with a large mass of wheat in the ration of swine toxicity will follow even in the presence of all the recognized factors for growth. Only in the presence of very liberal quantities of all these factors can the effect of the toxicity be overcome.

2. This toxicity manifests its action by producing important histological changes in the nervous system of the animal, not unlike those recorded for beri-beri.

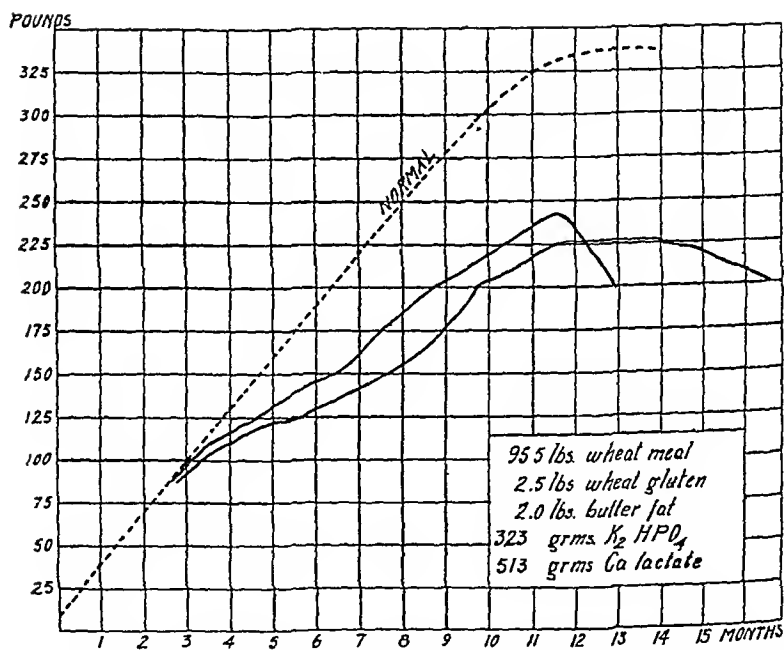
3. No one important factor for growth, such as better proteins, salts, or fat-soluble A, appears able to act as a complete corrective for this toxicity.

4. It also appears possible to produce similar pathological conditions in swine in the absence of all known toxic material and in the presence of a fair quality of protein, a plentiful supply of fat-soluble A, and water-soluble B, but a poor salt mixture; namely, that natural to the grains used.

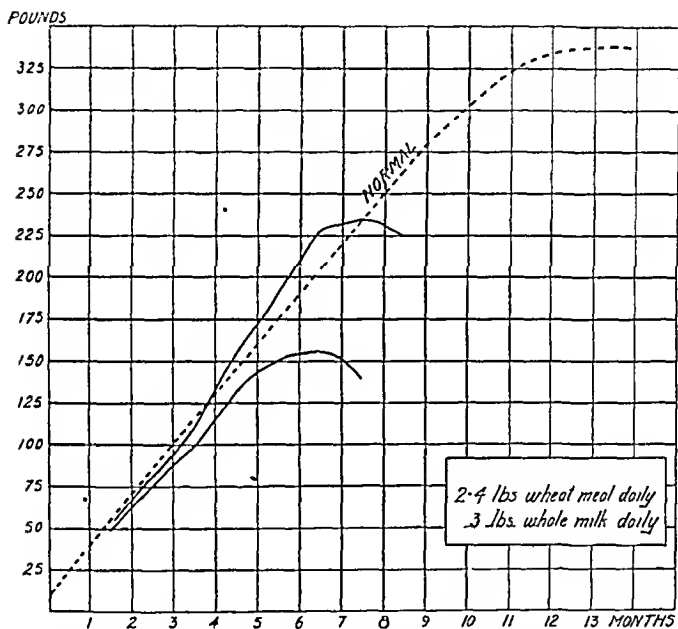
5. Excellent supplementary materials to the grains, even in the presence of the toxicity of the wheat products, have been found in alfalfa and commercial meat scraps. Probably milk if used in sufficient quantities would also serve this purpose admirably.

6. The factors introduced by alfalfa are undoubtedly an abundance of fat-soluble A and a better salt mixture. Its richness in calcium may be important and the relation of calcium to this problem will be studied further. With commercial meat scraps the factors for improvement are undoubtedly better proteins, more fat-soluble A, and a liberal supply of calcium phosphate resident in the bone material it carries.

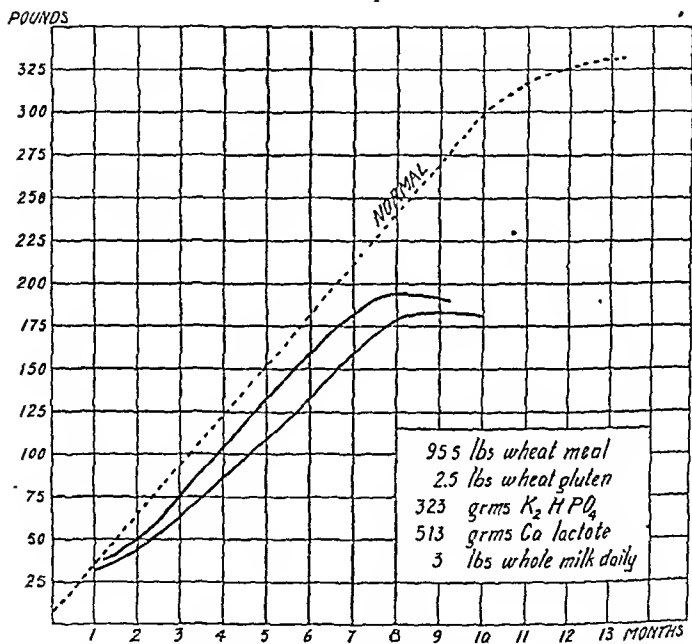




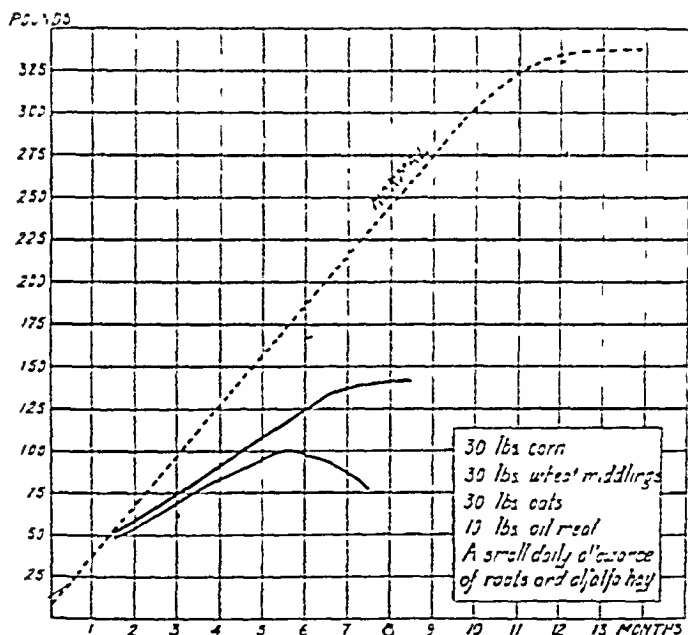
TEXT-FIG. 1. This shows the failure of swine to continue their growth on a ration of wheat, wheat gluten, butter fat, and salts.



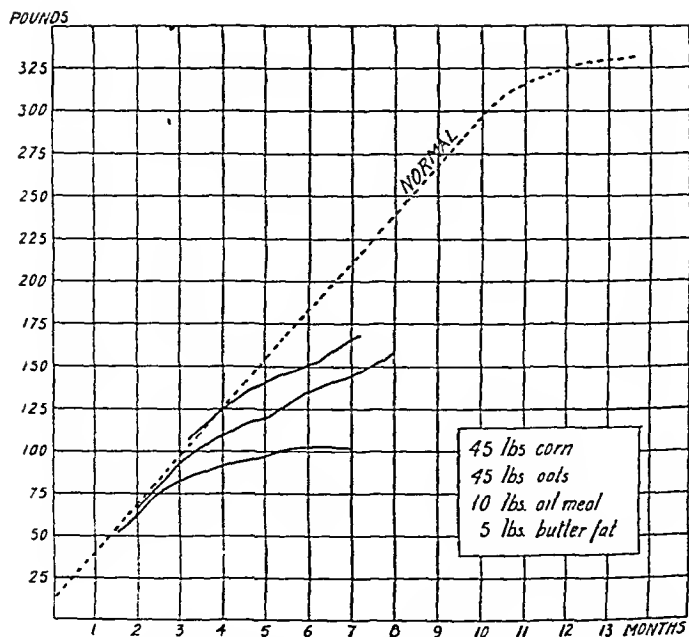
TEXT-FIG. 2. Growth curves of animals receiving wheat meal and 3 pounds of whole milk per individual daily. Growth was not sustained.



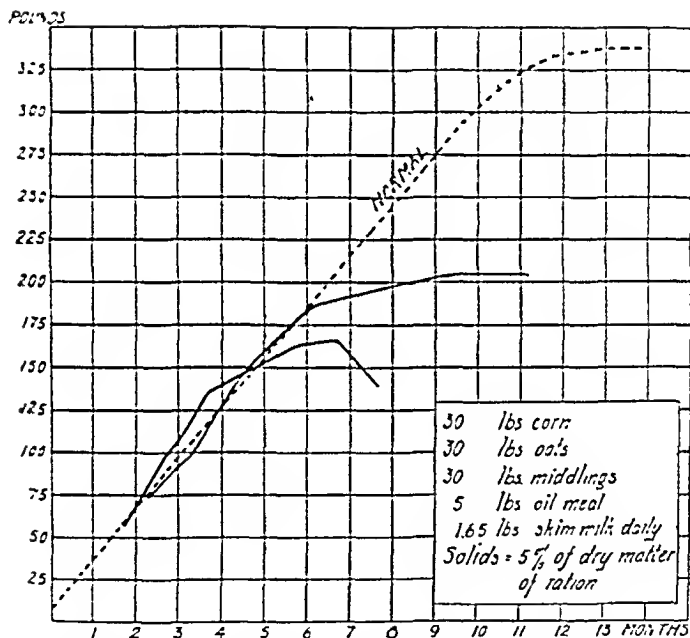
TEXT-FIG. 3. Growth curves of animals receiving wheat meal, wheat gluten, salts, and 3 pounds of whole milk daily per individual. Growth was not sustained.



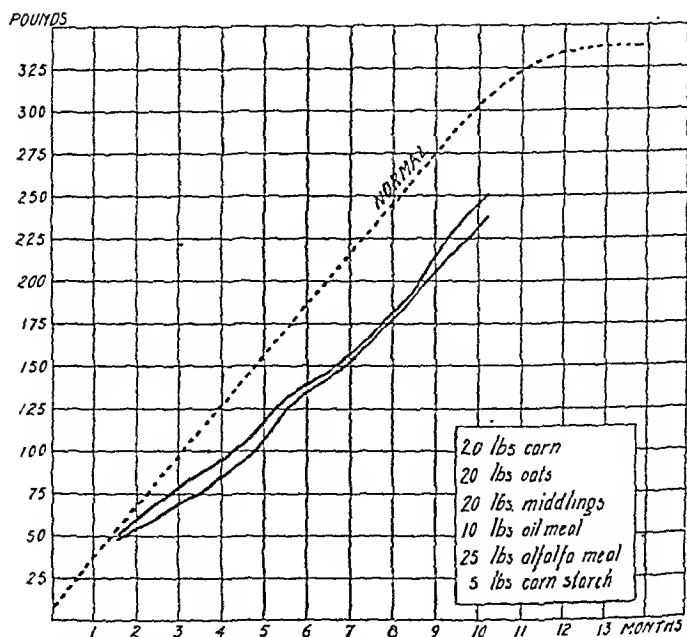
TEXT-FIG. 4. Growth curves of animals receiving a ration of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, 10 parts of oil meal, and a quantity of alfalfa and roots constituting less than 5 per cent of the dry matter of the ration. Normal growth was not continuous and the animals passed into the condition shown in Fig. 8.



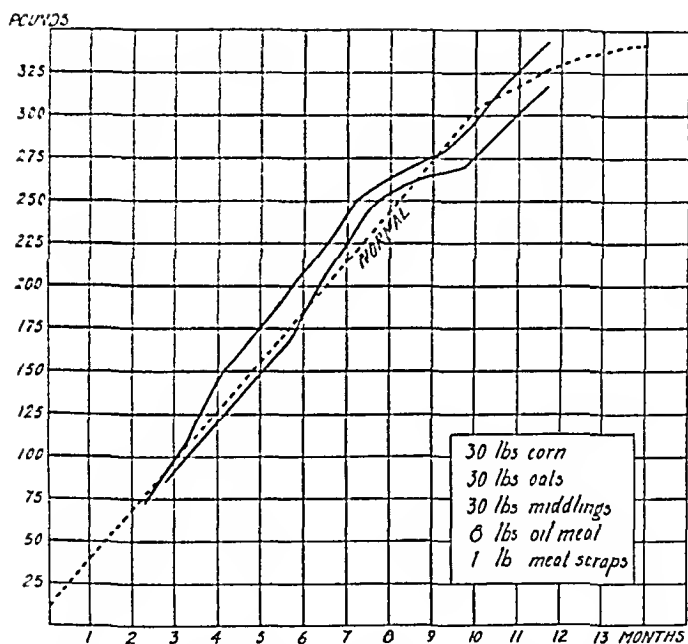
TEXT-FIG. 5. Growth curves of animals on a ration of 45 parts of corn meal, 45 parts of oats, 10 parts of oil meal, and 5 parts of butter fat. Growth was slow, below normal, and not sustained. These animals likewise passed into a poor condition. An insufficient salt mixture is probably responsible.



TEXT-FIG. 6. Curves of growth of swine receiving a grain mixture of 30 parts of corn, 30 parts of wheat middlings, 30 parts of oats, 5 parts of oil meal, and 1.65 pounds of skim milk daily. The mineral content of the ration and the quantity of fat-soluble A were probably insufficiently supplied to overcome the toxicity of the wheat middlings.



TEXT-FIG. 7. Curves of growth of swine on a ration made up of 20 parts of corn, 20 parts of oats, 20 parts of middlings, 10 parts of oil meal, 25 parts of alfalfa meal, and 5 parts of corn starch. The growth curve is a little below the normal, but physiological soundness was attained. The success secured here was probably due to a liberal supply of fat-soluble A and a better salt mixture, due to the introduction of the alfalfa meal.



TEXT-FIG. 8. Curves of growth of swine on a ration of 30 parts of corn, 30 parts of middlings, 30 parts of oats, 8 parts of oil meal, and 1 part of meat scraps. Normal curves of growth were obtained, but reproduction was not successful. The mothers conceived, developed the young, but they were born dead and hairless.





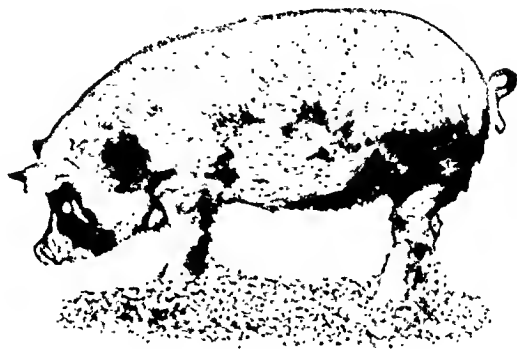


FIG. 1

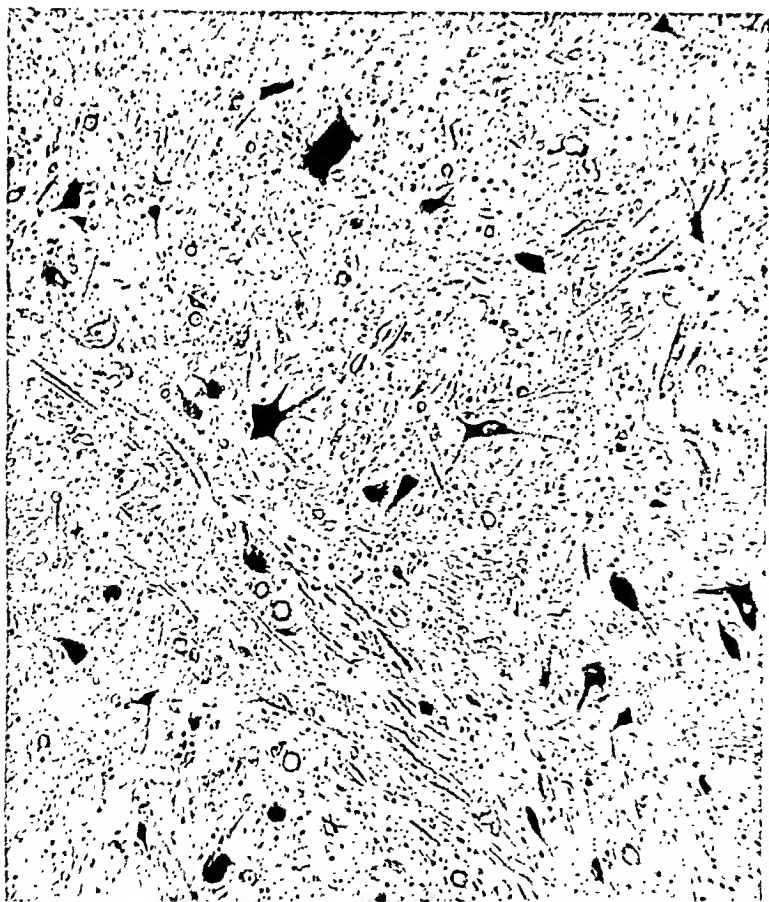






FIG. 5.





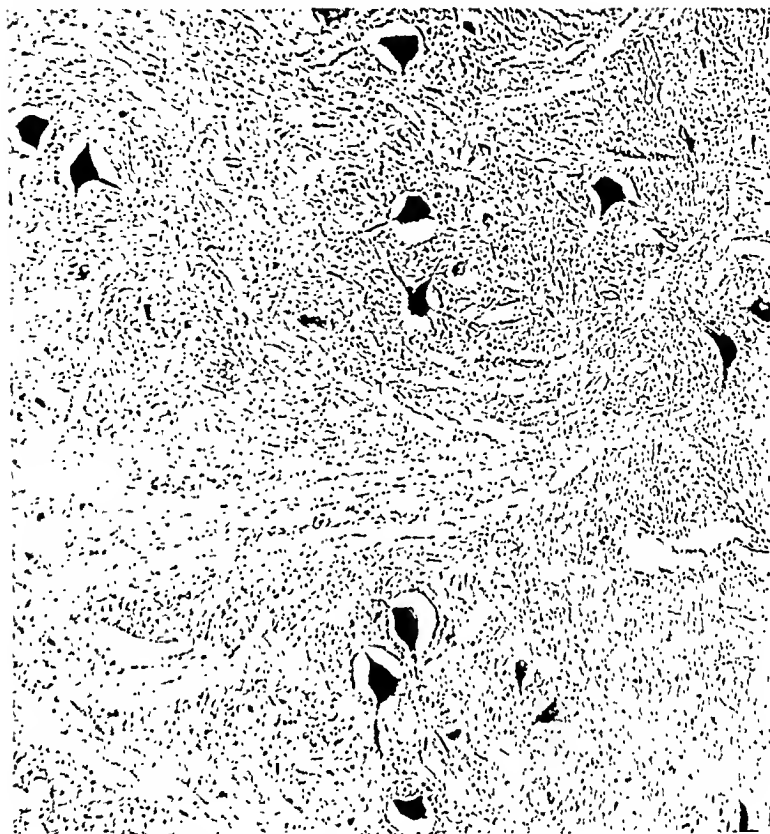
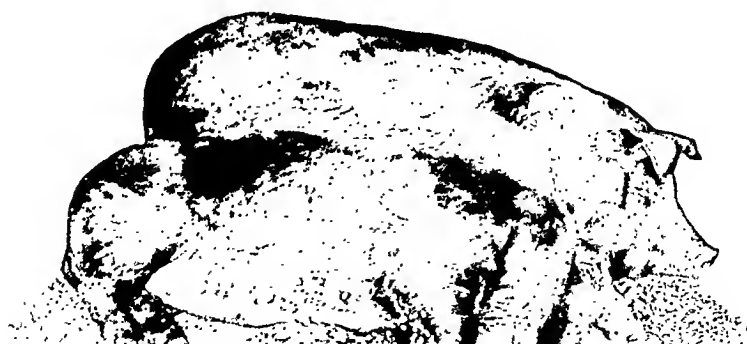


FIG. 3.





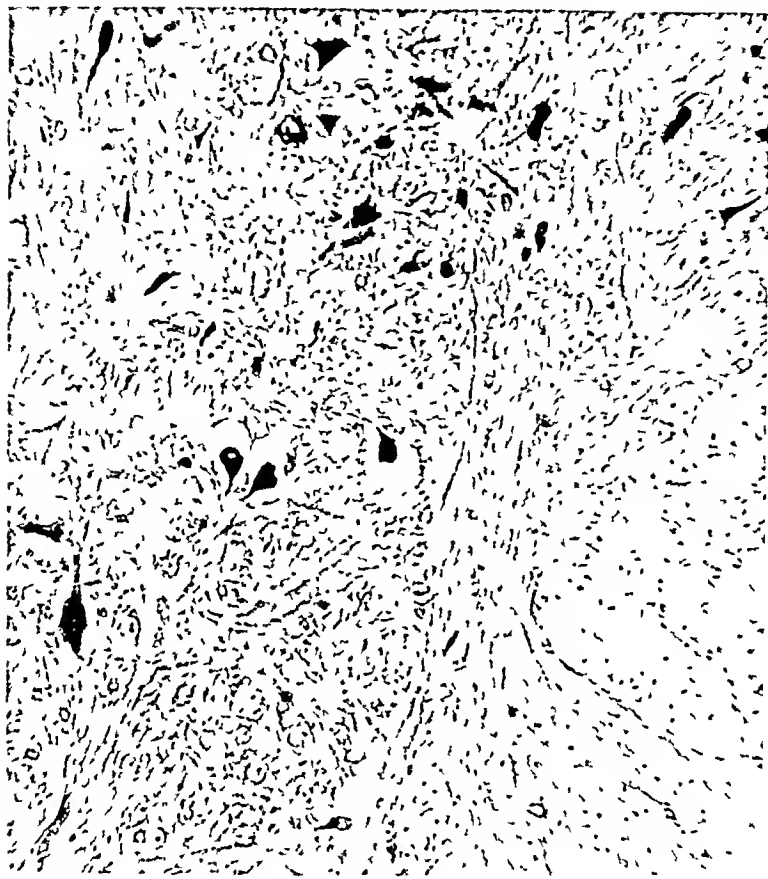


FIG. 5







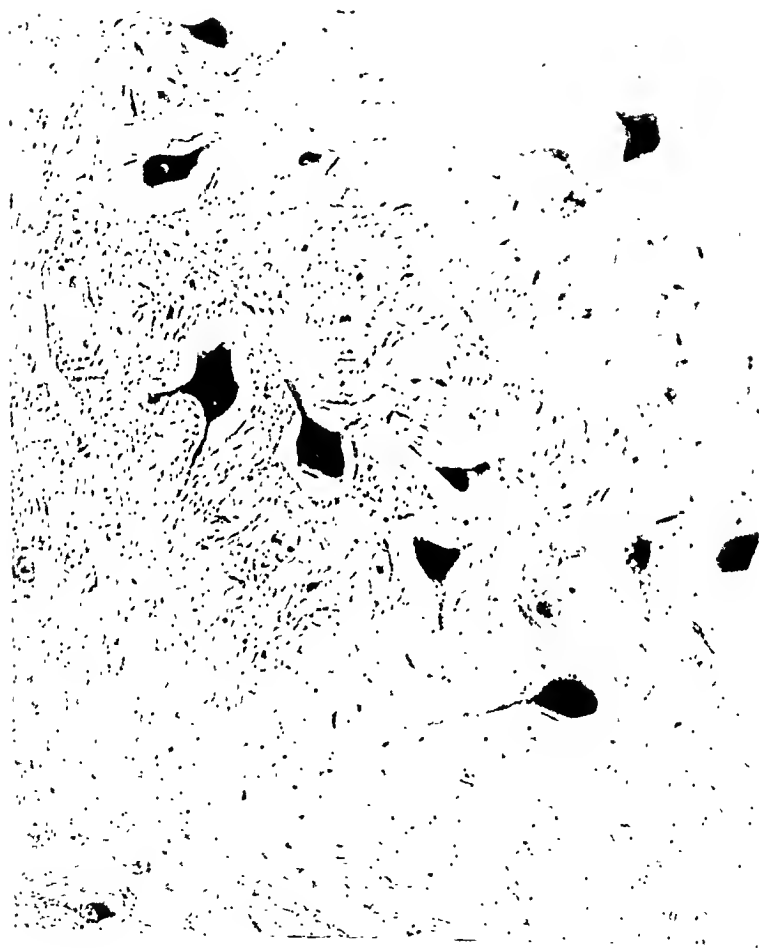


FIG. 7.

(Hart, Miller, and McCollum: Deficiencies of Wheat and *Secale*)





FIG. 8.



FIG. 9.

(Hart, Miller and McCollum Deficiencies of Wheat and Grain Mixtures



## STUDIES OF AUTOLYSIS.

### III. THE EFFECT OF REACTION ON LIVER AUTOLYSIS.

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(Received for publication, April 14, 1916.)

In previous papers on the subject of autolysis<sup>1</sup> we have presented evidence which appears to indicate that the accelerating effect of acids and acid salts on the autolyzing liver is due to a change in the proteins from the resistant type which constitutes the bulk of the liver tissue to a form which will digest. While looking for activation of an enzyme as one of the factors governing the autolytic process we have thus far failed to find direct proof of any such activation. Indeed the failure of certain foreign proteins to digest at a more rapid rate in the presence of acids which greatly increase the rate and extent of liver-protein digestion, is direct evidence to the contrary.

In this paper we wish to present further data upon the determinative effect of *reaction* in liver autolysis with its bearing on the mechanism which controls the direction and extent of autolysis under physiological and pathological conditions. The experiments may be grouped as studies of the effect of acidity, neutrality, and alkalinity upon the digestion of liver proteins and of certain foreign proteins.

#### *Methods.*

In some of the experiments tannic acid was used as the protein precipitant, as described in previous papers. In others we have employed trichloroacetic acid as suggested by Greenwald,<sup>2</sup> titrat-

<sup>1</sup> Bradley, H. C., and Morse, M., *J. Biol. Chem.*, 1915, xxi, 209. Bradley, H. C., *J. Biol. Chem.*, 1915, xxii, 113.

<sup>2</sup> Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

ing the filtrates with the formol method or estimating the amino nitrogen by the Van Slyke micro apparatus. The trichloroacetic-acid-formol method has the great advantage of rapidity over the tannic acid technique. Inasmuch as only amino-acid carboxyl groups are titrated, the method necessarily gives a slightly different picture of autolysis than the soluble nitrogen method. In a general way, however, the two methods have been found to parallel each other closely, and the results of titration accord well with the results obtained by the Van Slyke method. The filtrates are always colored slightly yellow so that some practice is required in getting the end-points. 25 cc. of the digest are mixed with 50 cc. of triehloroacetic acid and made up to 100 cc. The mixtures are filtered after standing over night. 0.2 N NaOH is used for titration with phenolphthalein as indicator. The figures in the tables representing digestion are cc. of 0.2 N NaOH per 25 cc. filtrates where not otherwise designated.

### *I. The Effect of Acids and Acid Salts.*

The liver proteins are very sensitive to acidity. In previous experiments the acidity of manganous salts, and of 0.01 N HCl, were shown to affect the rate and extent of autolysis markedly. We have here compared a number of other acids and salts.

1. CO<sub>2</sub>.—The gas, thoroughly washed through NaHCO<sub>3</sub> and toluene water, was bubbled slowly through the digesting mixtures. Autolysis increased. This confirms the work of Yoshimoto with CO<sub>2</sub>, boric, and salicylic acids.<sup>3</sup>

TABLE I.  
*Beef Liver.*

	Days.					
	0	1	3	6	7	11
I. Control.....	0.40	1.80	2.40	2.70	2.60	2.60
II. " + CO <sub>2</sub> .....	0.50	2.40	3.20	3.50	3.60	3.90

2. HCN.—The acid was prepared by several redistillations under diminished pressure from Merck's 5 per cent chemically pure

<sup>3</sup> Yoshimoto. S., *Z. physiol. Chem.*, 1908, lviii, 341.

acid. The original was contaminated with small amounts of the halogen acids and sulfuric acid and was found to give increased autolysis. The purified acid, whose strength was determined by silver precipitation, had no appreciable effect either upon the rate or the equilibrium of the digesting liver. HCN is known to be one of the weakest of the common acids. Made up with distilled water in strengths comparable to the concentrations used in the digests, it gave a  $H^+$  concentration of about  $P_H = 6.0 \pm$ , but was without measurable effect on the  $H^+$  concentration of a digest.<sup>4</sup>

TABLE II.  
*Horse Liver.*

	Days.				Gain.
	0	2	5	10	
I. Control.....	0 40	0 95	1 15	1 20	0 80
II. " + HCN 0.02 N	0 40	1 05	1 30	1 30	0 90
III. " + " 0 01 N	0 40	1 00	1 25	1 20	0 80
IV. " + " 0 005 N	0 40	0 95	1 20	1 25	0 85

3.  $H_3PO_4$  and  $KH_2PO_4$ .—Equivalent solutions of the salt and acid as measured by titration with NaOH give marked increases in autolysis. The acid shows the usual optimum concentration of about 0.04 N. The salt, on the other hand, even in 0.2 N strength, is less effective than the most dilute acidity tried—0.01 N. The  $H^+$  concentration in 0.01 N  $H_3PO_4$  is higher than in 0.2 N  $KH_2PO_4$ .

<sup>4</sup> The hydrogen ion determinations cited in this paper were made with the Sørensen indicator method as applied by Rowntree and his coworkers (Levy, R. L., Rowntree, L. G., and Marriott, W. McK., *Arch. Int. Med.*, 1915, xvi, 389). 5 cc. of the digest were dialyzed against 5 cc. of distilled water in steamed Jena glass for 3 minutes, using collodion bags. The phosphate solutions were corrected by titration but were not checked by the potentiometer, so that they are approximations rather than absolute figures.



TABLE III.

*Beef Liver.*

					Days.				Gain.
					0	2	6	12	
I.	Control				0.80	1.25	1.90	2.55	1.75
II.	"	+	KH <sub>2</sub> PO <sub>4</sub>	0.01 N	0.80	1.40	2.10	3.00	2.20
III.	"	+	"	0.02 N	0.80	1.45	2.40	3.35	2.55
IV.	"	+	"	0.04 N	0.80	1.70	2.60	3.60	2.80
V.	"	+	"	0.1 N	0.80	1.90	2.80	3.90	3.10
VI.	"	+	"	0.2 N	0.80	1.95	3.10	4.00	3.20
VII.	"	+	H <sub>3</sub> PO <sub>4</sub>	0.01 N	0.80	2.00	3.20	4.05	3.25
VIII.	"	+	"	0.02 N	0.80	2.30	3.60	4.50	3.70
IX.	"	+	"	0.04 N	0.80	2.75	3.90	4.90	4.10
X.	"	+	"	0.1 N	0.80	2.70	3.90	4.50	3.70
XI.	"	+	"	0.2 N	0.80	1.20	1.20	1.35	0.55

4. *Optimum Concentration of Various Acids.*—The results tabulated below<sup>5</sup> are in confirmation and extension of the work of Arinkin.<sup>6</sup> Within rough limits the strong acids accelerate best between concentrations of 0.04 and 0.02 N when added to a 20 per cent liver digest. The less easily dissociated acids require higher concentrations for maximum effects. The evidence therefore points to the H<sup>+</sup> concentration as being the factor of chief importance. As a general rule the optimum H<sup>+</sup> concentration in such liver digests appears to be about P<sub>H</sub> = 6.00, and filtrates or dialysates show a slightly acid reaction to Congo red.

TABLE IV.

Acid.	Optimum concentration.	Indicator reactions.
HCl	0.01–0.02 N	Congo red; slight.
H <sub>2</sub> SO <sub>4</sub>	0.02 N	" " "
H <sub>3</sub> PO <sub>4</sub>	0.04 N	" " "
KH <sub>2</sub> PO <sub>4</sub>	0.1–0.2 N	" " "
Acetic.	0.1 N	" " "
Lactic.	0.04 N	" " "

<sup>5</sup> For many of the data in this experiment we are indebted to Miss Katherine Wright of this laboratory, in whose graduation thesis they appear.

<sup>6</sup> Arinkin, M., *Z. physiol. Chem.*, 1907, liii, 192.

## II. The Effect of Neutral and Alkaline Reaction.

5.  $K_2HPO_4$ .—This salt is approximately neutral to litmus. In distilled water a 1 per cent solution has a  $H^+$  concentration of  $P_n = 6.85$ . It is thus seen to be not far from the neutrality of water. Where present in an autolyzing mixture it will act as a buffer salt tending to maintain a constant  $H^+$  level despite the normal formation of lactic acid in the tissue. Where small amounts are used the proportion of the acid phosphate formed will be sufficient to allow digestion to go on, and litmus acidity to develop within 24 to 48 hours. Where large amounts of the salt are added the inhibition of autolysis is marked. A concentration of 1 per cent is about as effective as that of 4 per cent.

TABLE V.  
*Beef Liver.*

	Days					Gain
	0	1	2	5	10	
I. Control	0 70	1 40	2 20	2 80	3 10	2 40
II. " + $K_2HPO_4$ 4 0 per cent .	0 70	0 90	1 30	1 40	1 70	1 00
III. " + " 2 0 "	0 70	0 90	1 30	1 40	1 70	1 00
IV. " + " 0 8 "	0 70	1 00	1 30	1 60	1 80	1 10
V. " + " 0 2 " . .	0 70	1 20	1 60	2 00	2 45	1 75
I. Control.....	0 40		0 95	1 15	1 20	0 80
II. " + $K_2HPO_4$ 2 0 per cent	0 40		0 70	0 80	0 75	0 35
III. " + " 0 8 "	0 40		0 80	0 85	0 80	0 40
IV. " + " 0 4 "	0 40		0 80	0 90	1 00	0 60
V. " + " 0 2 "	0 40		0 85	1 00	1 10	0 70
VI. " + " 0 08 "	0 40		0 80	1 20	1 20	0 80
VII. " + " 0 04 "	0 40		0 95	1 20	1 25	0 85

Noteworthy is the difference between the two beef livers used. One digested normally three times as fast and as far as the other. We have frequently observed such large individual differences between livers of the same species as well as between the livers of different species. It should be noted too that some digestion goes on in both cases even with a large excess of the buffer salt present. That the inhibition is not caused by a toxic effect of the salt molecule on the enzyme is clear from the fact that in-

creasing the concentration from 1 to 4 per cent does not increase the inhibition. The mechanism of inhibition seems rather to concern the suppression of developing acidity in the mixture.

6.  $\text{CaCO}_3$ .—The presence of this salt diminishes the speed of the reaction and lowers the point of equilibrium to a small extent. The insoluble carbonate will neutralize the developing lactic acid but will at the same time liberate an equivalent amount of  $\text{CO}_2$ . Since the digestion bottles remain corked, an acidity comparable to that of  $\text{CO}_2$  will be the result. As has been shown,  $\text{CO}_2$  bubbled into a normal digest where lactic acid is also developing unchecked will increase the digestion. In this case we find inhibition expressive of the substitution of the weaker acid in the digest.

TABLE VI.

*Beef Liver. Tannic Acid Method.*

	Days.					Gain
	0	1	3	5	10	
I. Control	1 70	2 40	3 10	3 60	3 90	2 20
II. " + $\text{CaCO}_3$ 5 gm	1 80	2 20	2 60	3 10	3 30	1 50

7.  $\text{MnCO}_3$  and  $\text{CuCO}_3$ .—Manganous salts themselves exert no inhibitory effect upon the digestion of liver, but on the contrary may accelerate it. Copper salts, however, cause complete loss of activity. In these digests the insoluble manganous carbonate behaves much like the calcium salt, depressing the reaction slightly. Copper carbonate, however, allows no digestion to go on. Soluble copper salts are quickly formed in the mixture, as is indicated by the color of filtrates.

TABLE VII.

*Pig Liver. Tannic Acid Method.*

	Days.			Gain
	0	3	6	
I. Control	1 40	5 10	5 50	4 10
II. " + $\text{MnCO}_3$ 5 gm	2 60	4 30	4 70	2 10
III. " + $\text{CuCO}_3$ 5 "	1 40	1 40	1 30	0 00

8. *Colloidal Fe(OH)<sub>3</sub>*.—The colloidal iron solution gave some acceleration in this case. The dialysate from it showed a H<sup>+</sup> concentration of  $P_H = 6.1$ , which is hardly sufficient to account for the acceleration. In any case the solution does not effectively neutralize developing acidity in any of the concentrations used.

TABLE VIII.

*Pig Liver. Tannic Acid Method.*

	Days.				Gain.
	0	2	4	10	
I. Control.....	0 90	2 82	3 70	4 40	3 50
II. " + 0.5 cc. solution..	1.10	3 40	4 45	5 20	4 10
III. " + 1.0 " " ..	1 10	3 75	4.80	6 00	4 90
IV. " + 5.0 " " . . .	1 15	3 60	4 90	4 90	3 75
V. " + 10.0 " " . . . . .	0 80	3 80	3 60	4 00	3 20

9. *ZnO*.—The oxide was boiled with several volumes of water to remove any traces of soluble alkali. In the digests it prevents the development of acidity. The mixture reacts neutral to litmus throughout. Digestion is almost completely stopped. The inhibition is not due to a toxic action of the zinc salts on the enzyme, since we have shown previously that zinc sulfate accelerates autolysis. As with the neutral phosphate, a slight autolysis eventually shows itself, although within a 5 day period it is too small to measure.

TABLE IX.

*Pig Liver. Tannic Acid Method.*

	Days.					Gain.
	0	1	3	5	10	
I. Control. ....	1.60	2.60	3.10	3 90	4 40	2 80
II. " + ZnO 5 gm.....	1.60	1.40	1 50	1.60	2.30	0 70

10. *NaHCO<sub>3</sub>*.—Autolysis is inhibited by this salt in proportion to the concentration. The CO<sub>2</sub> liberated probably has some effect in counteracting the alkalinity of the salt in the lower concentrations.

Casein was dissolved in NaOH and neutralized to litmus. The  $H^+$  concentration of this solution was found to be  $P_H = 6.47$ , or about that of normal liver digests after 1 or 2 days' autolysis.

TABLE XII.

*Pig Liver. Tannic Acid Method.*

	Days.					Gain.	Digestion of foreign protein.
	0	1	3	5	10		
I. Control. ....	1.70	2.40	3.10	3.60	3.90	2.20	—
II. " + $CaCO_3$ 5 gm. ....	1.80	2.20	2.60	3.10	3.30	1.50	—
III. " + casein. ....	2.00	7.10	8.60	9.00	10.10	8.10	5.90
IV. " + { casein. .... $CaCO_3$ 5 gm. ....	2.00	6.20	7.60	8.00	8.80	6.80	5.30
V. " + peptone. ....	3.10	6.40	7.50	7.60	8.60	5.50	3.30
VI. " + { peptone. .... $CaCO_3$ ....	3.00	6.30	7.50	8.30	9.00	6.00	4.50

There appear to be a very slight inhibition of casein digestion and an increased digestion of peptone. If the figures are analyzed to determine the rate of change, it will be found that casein digests more slowly during the first 24 hours in the presence of the carbonate, while the peptone digests more rapidly. Under the same conditions the liver digests at about half its normal rate. The experiment shows a lack of uniformity in the effect produced upon the three digestible proteins—liver, casein, and peptone. This lack of uniformity will be found throughout the experiments which follow and constitutes a strong argument against activation of the enzyme as a determinative influence in autolysis.

13. *Peptone with ZnO and Alkalies.*—In this experiment liver digestion is reduced to about 25 per cent of its normal figure in the presence of ZnO; the digestion of peptone to about 60 per cent. In the presence of MgO liver is reduced to 20 per cent of the normal, while peptone is reduced to about 45 per cent. Where there was insufficient  $Na_2CO_3$  to maintain litmus neutrality throughout the autolysis, liver digestion was reduced to 50 per cent of the normal while peptone digested to 90 per cent of the normal.

TABLE XIII.

*Beef Liver. Tannic Acid Method.*

	Days					Gain	Digestion of foreign protein.
	0	1	2	5	0		
I. Control...	1 60	2 60	3 10	3 90	4 40	2 80	—
II. " + ZnO	1 60	1 10	1 50	1 60	2 30	0 70	—
III. " + MgO	1 60	1 70	1 10	1 50	2 20	0 60	—
IV. " + peptone	2 80	6 10	6 70	7 90	9 00	6 20	3 40
V. " + {peptone ZnO	2 80	1 00	1 20	4 60	5 60	2 80	2 10
VI. " + {peptone MgO	2 70	1 10	4 00	1 00	1 70	2 00	1 40
VII. " + Na <sub>2</sub> CO <sub>3</sub>	1 60	1 90	2 00	2 60	3 00	1 40	—
VIII. " + {peptone Na <sub>2</sub> CO <sub>3</sub>	3 00	6 00	5 90	6 10	7 40	4 40	3 00

Again if we determine the rate it is found that in the first 24 hours there is no digestion of liver with ZnO and MgO. Under the same conditions peptone digests at 50 per cent of its normal rate. With the carbonate present the rate of liver digestion is about 30 per cent of the normal, while the peptone digests at full normal rate.

14. *Casein with ZnO and MgO.*—In the presence of the oxides, liver shows no digestion in a 5 day period. With ZnO casein is reduced to 30 per cent of its normal rate in the first 24 hours, with MgO to 17 per cent. The rate of liver digestion is zero. In order to eliminate the error which might result from hydroly-

TABLE XIV.

*Beef Liver. Tannic Acid Method.*

	Days				Gain	Digestion of foreign protein
	0	1	3	5		
I. Control	2 20	4 70	5 55	5 60	3 40	—
II. " + ZnO	2 20	2 20	2 00	2 20	0 00	—
III. " + MgO	2 20	2 40	2 40	2 20	0 00	—
IV. " + casein	2 30	8 80	9 50	9 80	7 50	4 10
V. " + {casein ZnO	2 30	3 50	3 90	4 30	2 00	2 00
VI. " + {casein MgO	2 30	3 00	3 15	3 10	0 90	0 90

sis of the foreign proteins by the alkali alone we have digested casein and peptone with ZnO and MgO at the same temperature and for the same length of time. No hydrolysis occurs.

15. *Casein and NaOH*.—Progressive additions of NaOH produce the graded inhibition of liver autolysis reported in Table XI. The effect of the alkali upon casein digestion is quite otherwise. Even in the higher concentrations of the hydroxide casein digests better than in the normal control.

16. *Peptone and NaOH*.—In a single experiment sufficient NaOH was added to reduce liver autolysis to 60 per cent of the normal. Peptone digested slightly better than in the normal.

17. *Casein and K<sub>2</sub>HPO<sub>4</sub>*.—Adding progressive amounts of the salt, as in Table V, with and without casein, we find parallel inhibition of both liver and casein digestion.

18. *Casein and NaHCO<sub>3</sub>*.—With varying amounts of the salt, sufficient to reduce liver digestion to less than 50 per cent of the normal amount, casein digested better than in the normal, except in the highest salt concentration of 2 per cent.

19. *Gelatin and NaHCO<sub>3</sub>*.—The digestion of gelatin which occurs in the normal liver mixture is inhibited by the addition of the salt, but to a less extent.

TABLE XV.

*Beef Liver.*

		Days.					Gain.	Digestion of gelatin
		0	1	3	5	10		
I.	Control.....	0.50	2.30	2.35	3.10	3.40	2.90	—
II.	" + gelatin.....	0.80	3.30	5.10	5.25	5.75	4.95	2.05
III.	" + NaHCO <sub>3</sub> 2 gm...	0.50	1.30	1.60	1.65	1.85	1.35	—
IV.	" + {gelatin..... NaHCO <sub>3</sub> 2 " ..	0.80	2.30	3.25	—	3.80	3.00	1.65
V.	" + NaHCO 5 " ..	0.50	1.10	1.30	1.60	1.50	1.00	—
VI.	" + {gelatin..... NaHCO <sub>3</sub> 5 " ..	0.80	2.10	2.75	2.95	3.25	2.45	1.45

It is of interest to note that in this liver gelatin digests at the identical rate in the three mixtures for the first 24 hours. At the beginning of autolysis, therefore, the rate was the same

whether the salt was present or not. The following analyses of the data will illustrate this point:

Control gains 1.80 cc. during the first 24 hours.

Control + gelatin gains 2.50 cc.

Gelatin alone (difference) 0.70 cc.

Salt mixture (2 gm.) gains 0.80 cc.

Salt mixture + gelatin gains 1.50 cc.

Gelatin alone 0.70 cc.

Salt mixture (5 gm.) gains 0.60 cc.

Salt mixture + gelatin gains 1.30 cc.

Gelatin alone 0.70 cc.

This point we wish to emphasize because it clearly shows that the normal and alkaline liver mixtures are equally active as judged by the speed of gelatin digestion during the early period of the reaction, when differences in speed would be readily recognized. Since the amount of gelatin added was the same in each case, equal rates of digestion indicate equal amounts of enzyme, or equal activity of enzyme. As far as the experiment goes it tends to negate a change in activity of the enzyme which digests gelatin, even in the presence of considerable bicarbonate. Since gelatin is a rather non-specific type of protein, digesting in most protease solutions, it seems fair to conclude that the same enzyme which digests the gelatin also digests the liver proteins. If this assumption is correct, there is no indication of loss of enzyme activity even at the low  $H^+$  level of this experiment.

20. *NaOH and Hemoglobin.*—Dog hemoglobin was found to digest slowly in an autolyzing liver. The addition of NaOH (25 cc. 0.2 N solution in 250 cc. liver mixture) inhibited the digestion of liver and of hemoglobin equally. A powdered fibrin preparation was found not to digest in either case.

In summarizing the effect of varying reaction of the liver mixtures upon the digestion of foreign proteins, it is clear that no parallelism has been found. Digestion of liver may be almost completely stopped by a salt which even accelerates the digestion of casein by the liver. Peptone may digest at its normal rate in a liver which is reduced to half its normal speed of autolysis



by the reaction. Gelatin may digest at normal speed in a mixture which reduces the speed of liver digestion to a third of the normal. Hemoglobin may show equal inhibition with liver. Acids which cause liver to digest much more rapidly and completely do not alter the rate of digestion of casein and peptone. Such variations do not indicate such a common factor as the activity of an enzyme. They rather indicate that the various proteins used, including the liver proteins, may or may not be altered by the presence of acids or salts or alkalies in the same direction or to the same extent.

From the spleen Hedin<sup>11</sup> isolated two enzymes,  $\alpha$  and  $\beta$  proteases, active in alkaline and acid media respectively. If we assume that the liver contains these enzymes in significant amounts also, then it is apparent that the  $\alpha$  protease is of no moment in the autolysis, since under conditions of neutrality or alkalinity there is practically nothing to digest. Nor does the possible activation of the  $\beta$  protease appear at all determinative in autolysis since the enzyme appears to be fully active at a  $H^+$  level at which there is little or no available protein substratum. The effect of reaction upon liver autolysis may be broadly stated as follows:

1. The progressive addition of alkali, or of salts like  $NaHCO_3$  and  $K_2HPO_4$ , reduces the normal rate and extent of liver autolysis in rough proportionality to the amount added, until complete or nearly complete inhibition results.

2. The progressive addition of acids and acid salts leads to an increase in the rate and extent of liver autolysis in rough proportionality to the amount added.

3. In progressive alterations of reaction from faint alkalinity to litmus to an acidity which may be just recognized by Congo red, and in these experiments amounts to a 0.04 N solution, we may pass from zero digestion (or a low minimum figure) to 90 per cent of the total protein of the tissue, and this would approximate 100 per cent digestion if the connective tissue fraction could be eliminated.

4. Such progressive alterations of the speed of digestion and the point of equilibrium do not appear to be correlated with any cor-

<sup>11</sup> Hedin, S. G., *J. Physiol.*, 1903-04, xxx, 155.

responding change in enzyme activity, as measured by the digestion of foreign proteins.

5. Such progressive alterations of speed and equilibrium are of just the type produced by progressive changes in the mass of substratum in the digest.

We therefore conclude that the effect of reaction in a liver digest is to alter the liver proteins, increasing or decreasing the mass of available substratum as the reaction becomes more or less acid. Soon after death the normal liver is found to give a dialysate with a  $H^+$  concentration averaging about  $P_H = 7.00$ . At the moment of death it is probably much lower and approximates the reaction of blood,  $P_H = 7.40-7.80$ . At these levels little or no autolysis takes place. Unless prevented, the development of acid is very rapid and produces a rapid rise in the  $H^+$ , as Morse has recently shown.<sup>12</sup> Such an increase will determine the amount of actual substratum that becomes available in the normal liver for autolytic proteolysis. We feel confident in asserting that the variations which one finds in the normal autolysis of various livers will be found to correspond to the amount of acid produced. For example, the liver of a rabbit is as a rule slow to autolyze and reaches a low point of equilibrium normally. The liver of a dog, on the other hand, digests more rapidly and to a higher amino-acid level. The individual differences can be made to disappear, however, by artificially controlling the acidity. Thus rabbit liver may be made to digest like dog liver by adding the proper amount of acid, and dog liver can be reduced in its digestion to that of rabbit liver by the addition of the proper amount of  $K_2HPO_4$ . The rabbit liver probably produces on the average less acid than does that of the dog. The extent of individual variations in the same species may be quite large. In Fig 1 are shown the limiting variations and the mean of eight dog livers—all apparently normal. Seven of the eight curves fell within the limits of 1 and 2. The curve of the eighth dog liver is No. 3 and is evidently abnormal, though the dog was apparently perfectly normal and the liver, to outward appearances, was so.

<sup>12</sup> Morse, M., *J. Biol. Chem.*, 1916, **xxiv**, 163

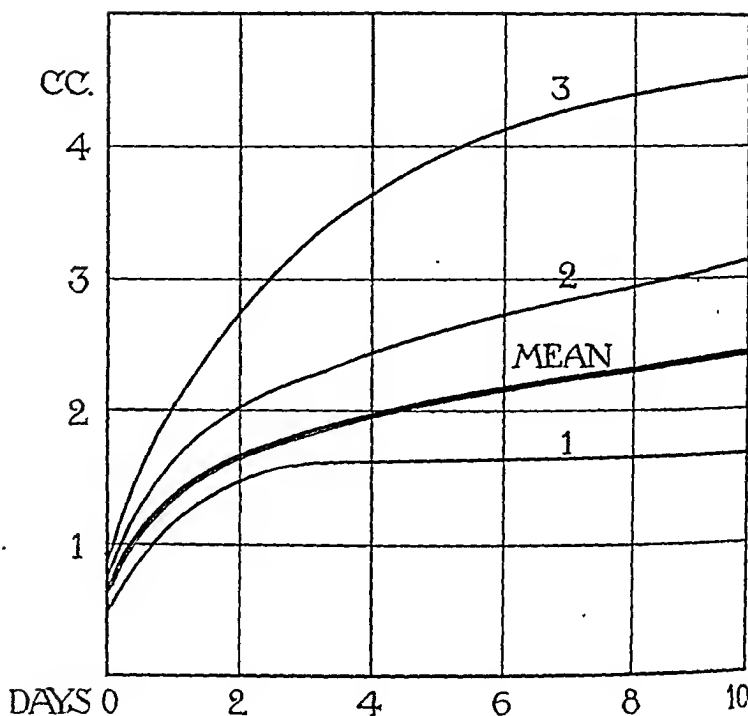


FIG. 1.

The shift from non-available proteins to available is evidently a reversible reaction depending for its equilibrium upon the  $H^+$  concentration of the cell at the given moment. The non-available protein fraction may be a protein salt, or a polymerized protein aggregate of large size. It is well known that many polymerizations are facilitated by an alkaline reaction. The available protein fraction may be a dissociated protein salt, or a more highly dispersed colloid. The charge on the colloid particles may determine availability. Without wishing to commit ourselves as to just what molecular changes are involved in the shift from one type to the other with a change in the  $H^+$  concentration, we wish to illustrate by the following diagram what we conceive to be the relationships existing in a liver cell.

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acids would increase within the cell and diffuse into blood and lymph. It is well known that nitrogen elimination is increased in severe acidosis.

During starvation the acidosis which appears after a variable fore-period, and is believed to be associated with imperfect fat combustion, is correlated with increased nitrogen elimination. The mobilization of tissue proteins in the form of amino-acids may indeed depend upon; or be facilitated by this shift in the salt balance of blood and tissues toward a higher  $H^+$  level. Schryver<sup>13</sup> points out that the loss in weight by the liver and spleen during starvation is unusually large. Both of these organs are very sensitive to a change in the  $H^+$  concentration, as shown by autolysis.

In local areas diminished blood supply leads to partial asphyxia, poor removal and neutralization of  $CO_2$  and acid products of incomplete combustion. In such local areas we find atrophy, shrinkage, or vacuolation of the cells, changes in the staining reactions—all expressive of autolysis and associated with the shift in the available proteins of the cell caused by shift in acidity. Such atrophy may be induced experimentally by ligation of arteries, by embolism, or by thrombosis.

The presence in the cell of any substance preventing the normal oxidations should lead to the formation of intermediate acid products. Phosphorus, a powerful reducing agent and itself oxidizing under certain conditions to a strong acid, is well known for its disintegrating effect upon the liver.<sup>14</sup> We believe that part of the effectiveness of phosphorus in producing rapid degeneration of the liver is due to the production of small amounts of phosphoric acid within the cells themselves, as well as to the prevention of normal oxidations of the cell substances so that the production of organic acids results. At least it is suggestive that phosphorus has little accelerating effect on a liver autolysis unless oxygen be admitted to the digest. We expect to present further data on this point later.

Graham<sup>15</sup> believes that chloroform poisoning results in the formation of  $HCl$  within the liver cells with a rise in the  $H^+$  con-

<sup>13</sup> Schryver, S. B., *Biochem. J.*, 1906, i, 123.

<sup>14</sup> Jacoby, M., *Z. physiol. Chem.*, 1900, xxx, 174.

<sup>15</sup> Graham, E. A., *J. Exp. Med.*, 1915, xxii, 48.

tent as shown by indicators. Such a rise would increase the available substratum and cause autolysis. Acute atrophy of the liver is characteristic of chloroform poisoning.

Pressure on an organ leads to cutting down the blood and lymph flow within the compressed area. Partial asphyxia in such an area leading to the accumulation of acid products would thus increase autolysis and lead to the well known pressure atrophies. Pressure and alteration of the blood supply probably cause the atrophy of the mammary gland after lactation, by the same mechanism.

Alterations in the blood supply of the uterus after parturition probably cause a shift toward acidity in the cells and thus a change in the proteins resulting in the rapid atrophy of that organ. Although we have not as yet studied the effect of increased acidity on the autolysis of the uterus it will in all probability be found to react as does the liver, though to a less marked degree.

In the rapidly growing tumors, especially carcinomata, we find the mass of cells outgrowing the blood supply, or by compression changing it to certain areas of the tumor. Such rapidly growing tissues are especially liable to necrosis and extensive autolysis. The onset and the extent of this autolysis is probably determined by partial asphyxia of masses of cells, accumulation of acid products, the shift in the proteins, and their consequent digestion.

Hypertrophy itself seems, as a rule, to be conditioned by an increased blood supply. Such an increase would insure complete oxidation, removal of  $\text{CO}_2$ , neutralization of organic acids if any formed, and a constantly renewed supply of the phosphate mixture of the blood. The salt balance of the cells would therefore tend to remain approximately that of the blood, with a  $\text{H}^+$  concentration<sup>4</sup> of  $\text{P}_n = 7.4-7.8$ . Such a condition would reduce autolysis to a minimum by converting all the available proteins into the reserve type. If more of the available type were synthesized more of the reserve fraction would accumulate. The tendency would therefore be to increase the cell proteins to the maximum, and the result would be a simple hypertrophy. In certain tissues this might be accompanied by cell division and a numerical hypertrophy as well. One of the important factors limiting this process of growth or hypertrophy will be the relation of the tissue to its blood supply—governed automatically by

the  $H^+$  level maintained in the tissue and its effect on the active masses of substratum in the cells. A point is eventually reached where further additions to the tissue mass must constitute an excess of demand over supply of oxygen, or mechanically prevent adequate movement of lymph and blood. A condition of equilibrium is the result, such that further tendency to growth is counterbalanced by a rising of the  $H^+$  level in some of the cells least favorably located in relation to the blood, with consequent tendency toward autolysis, and shrinkage.

Hypertrophy, atrophy, and maintenance of tissue mass are, we believe, governed by some such fundamental mechanism as we have described. Innumerable complicating factors must intervene to modify the final condition of a tissue, its rate and direction of change; but it seems likely that in all such tissues as are sensitive to increased acidity and respond by increased autolysis, the determining mechanism is the one described above for the liver.

#### SUMMARY.

1. Progressive changes in the reaction of autolyzing liver tissue, from an alkalinity expressed by  $P_H = 7.4$  to the optimum acidity of  $P_H = 6.0$  (measured in the dialysate), cause proportionate changes in the rate and extent of autolysis from nearly zero to 90 per cent digestion.

2. Under these same conditions of varied reaction certain foreign proteins digest without indicating any change which could be interpreted as an activation of an enzyme.

3. The effect of such changes in the reaction of the tissue is to alter the mass of digestible protein present in the liver from nearly zero, when alkaline, to nearly complete digestibility at an acidity of  $P_H = 6.0$ .

4. A tentative working hypothesis based upon these facts is presented and applied to a number of well known conditions which involve autolysis or the reverse process of hypertrophy in the living organism.

5. Atrophy, involution, necrosis, hypertrophy, and tissue equilibrium in such tissues as respond to increased acidity by increased autolysis, appear to depend upon the mechanism described as of fundamental importance in determining the direction, rate, and extent of tissue change.

## STUDIES IN BENCE-JONES PROTEINURIA.

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(Received for publication, April 11, 1916.)

The subject of these studies is an intelligent patient under the care of Dr. C. H. Schoff, of Media, Pa., who supplied the following summary of the history.<sup>1</sup>

J. E. L. Age 52. White. Male. Childhood unimportant. When 26 he began to lose weight and after a short period of ill health had a hemorrhage from the lungs. After 2 years in the West he entirely regained weight and health. 4 years ago he commenced having attacks of bronchitis accompanied by severe shooting and aching pains in the back and legs. The last severe attack of these pains commenced in February, 1915, and continued for several months. In April of that year Bence-Jones protein was first discovered in the urine. There were at times cough and expectoration, but never tubercle bacilli in the sputum. A few of the latter were at one period said by another observer to be found in the urine, but this could never afterwards be confirmed. Marked anemia, red corpuscles 2,000,000, and hemoglobin 40 to 50 per cent. Great prostration. Weight reduced from a normal of about 150 pounds to 120 pounds. Considerable proteinuria. Since last autumn the patient has gradually improved, and at present, March 1, 1916, his weight has become practically normal; red corpuscles 4,000,000. He has now no acute pain in the back or legs, but sometimes slight aching and stiffness. He can walk some distance, but still has a feeling of weakness. Protein excretion still marked, hyaline and a few granular casts, not much improvement of vision (diagnosis by a specialist, paralysis of optic nerve). X-ray findings both of spine and long bones have always been entirely normal.

The urine has been uniform, of normal appearance except for the exaggerated foam formation upon slight shaking, and with marked acid reaction (litmus). The total protein was weighed from time to time and has also been rather constant, fluctuating between 23 and 30 gm. daily.

<sup>1</sup> A discussion of the clinical features of this case will be published shortly by Dr. Schoff.



*Heat Reactions.*—When the urine at natural reaction is heated coagulation commences at about  $55^{\circ}$ , and proceeds rapidly as the temperature passes this point. There is no apparent tendency to decrease until the boiling point is almost reached ( $97^{\circ}$  or  $98^{\circ}$ ) when a slight lightening of the density is sometimes to be noticed. As the liquid begins to boil, further clearing is observed, and when boiling has continued for a few seconds nearly all the protein is redissolved. Upon cooling, the protein commences to reprecipitate as soon as the temperature has been lowered a few degrees, and finally becomes as dense as before; this can be repeated indefinitely. It is clear that we have to do with the so called Bence-Jones protein. In order to observe the above in typical form it is necessary to dilute the urine, which is best done by adding normal urine. If the dilution is made with water the protein will often redissolve badly unless a salt or, better, a few crystals of urea<sup>2</sup> are added. If the urine is heated at its natural concentration some of the protein will be precipitated in elastic clumps (they can be drawn out to form bundles of coarse threads), which tend to adhere to the sides of the vessel or float on the surface and do not again dissolve. These clumps have also been described by Magnus-Levy.<sup>2</sup> When the Bence-Jones urine is diluted the precipitate is fine and milky, going in and out of solution with ease.

The addition to the native urine of one drop of 50 per cent acetic acid to 10 cc. of urine, prevents the formation of the clumps and causes the solution at boiling and reprecipitation on cooling to be much more clearly manifested. That this may be associated with some such action as acid-albumin formation is suggested by the following experiment.

We heated in the water bath at  $52^{\circ}$  for 10 minutes urine to which had been added one drop of 20 per cent acetic acid for each 10 cc. In order to obtain a maximum surface and so insure prompt heating, the urine was divided among a number of test-tubes. No apparent change was produced.

The temperature of the bath was then raised to  $56^{\circ}$  for 10 minutes, with the result of a heavy white curdy precipitate. This was filtered off, and of this filtrate, A, one part was diluted with three parts normal urine and heated over the flame. This produced a heavy white opacity which largely disappears on boiling, and upon the addition of some urea almost entirely

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<sup>2</sup> Magnus-Levy, A., *Z. physiol. Chem.*, 1900, xxx, 200.

disappears; in either case it reappears when the liquid cools, and again disappears upon boiling.

The remainder of the filtrate A was put back in the water bath at 60° for 10 minutes. Result, heavy white curdy precipitate. It was filtered, and of this filtrate, B, one part was diluted with three parts normal urine and heated over the flame. This produced a moderate white opacity, which disappeared almost entirely upon boiling and reappeared on cooling.

The remainder of the filtrate B was again heated in the water bath, this time at 61° for 12 minutes. Result, moderately heavy white curdy precipitate. This was filtered, and of this filtrate, C, a portion diluted as before was heated over the flame. This produced a slight opacity, which disappeared almost entirely upon boiling, and reappeared on cooling.

The remainder of filtrate C was heated in the bath at 68° for 15 minutes. Result, slight white curdy precipitate. This was filtered, and of this filtrate, D, a portion without dilution was heated over the flame. The result was a slight opalescence, part of which disappeared upon boiling and returned on cooling.<sup>3</sup>

The rest of filtrate D was heated in the bath at 72° for 15 minutes. Result, slight opalescence. The filtrate, which even after repeatedly passing through the filter remained opalescent, was heated without dilution over the flame. Result, no change.

Under the conditions of the above experiment there is a continued precipitation from the temperature at which the Bence-Jones protein commences to come out up to the coagulation point of serum protein. This may be explained either by assuming that the Bence-Jones protein is composed of various fractions with different coagulation points, at least with different rapidity of precipitation; or, on the other hand, that the continued action of the acid had resulted in gradual change of the order of acid-albumin formation. We therefore made the following experiment.

Four tubes containing, respectively, 10 cc. of urine, 10 cc. of urine + 1 drop of 20 per cent acetic acid, 10 cc. of urine + 2 drops of acid, and 10 cc. of urine + 3 drops of acid, and a control tube of 10 cc. of nephritic urine (rich in albumin but apparently free from Bence-Jones protein) + 1 drop of the above acid, were placed in a water bath at 60° for 1½ hours. The Bence-Jones tubes commenced to precipitate in the order 2, 3, 1, 0 (referred to by the acid content), following each other at intervals of 15 seconds to 1 minute. Soon all showed a heavy white curdy precipitate, the tubes appear-

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<sup>3</sup> In order to make a certain judgment with these slight degrees of opalescence, the tube of urine is brought to boiling and then, avoiding shaking, the lower end (2 cm.) is held under the cold water tap, which permits of easy comparison between the hot and cold fluid.

ing almost solid. The control tube clouded slightly, and finally showed a very slight flocculent precipitate. At the end of the time the contents of all of the tubes were filtered and the respective filtrates heated over the flame. The filtrate from Tube 0 showed only slight opalescence, which was hardly affected by boiling. The same was true of Tube 1, although this was slightly decreased by boiling. These two filtrates also gave only moderate reactions with the Roberts test. The filtrate from Tube 2 showed marked opalescence, which nearly all disappeared upon boiling. The filtrate from Tube 3 gave a much heavier opalescence amounting almost to opacity, and this also was nearly all redissolved upon boiling. In both of these the original density returned on cooling. The filtrate from the control tube gave a considerable precipitate which showed no tendency to redissolve upon boiling.

It seems evident, therefore, that heating the Bence-Jones protein with even very moderate quantities of acetic acid is capable of effecting a change in the nature of the protein such that the temperature at which it coagulates is raised; but this in no way interferes with its characteristic behavior in regard to going into solution by boiling and reprecipitating on cooling.

*Salt Reactions.*—At the natural reaction of the urine one volume of saturated solution of ammonium sulfate added to one volume of urine causes incomplete precipitation, while two volumes cause complete precipitation.

Saturation with magnesium sulfate causes a considerable but not complete precipitation. This is opposed to the findings of most other writers, but Ville and Derrien<sup>4</sup> found that saturation with magnesium sulfate precipitated all the protein. When the urine is strongly acidified with acetic acid all the protein is precipitated by adding three volumes of a saturated solution of magnesium sulfate to one volume of urine.

If the urine at natural reaction be saturated with sodium sulfate, a large but not complete precipitation occurs. If a twice cold saturated solution of sodium sulfate be made up with hot water and allowed to cool almost to room temperature, it will be some time before the excess crystallizes out; if before this takes place one volume of the solution is added to an equal volume of urine containing a considerable amount of acetic acid, the precipitation is complete. Ammonium sulfate has the tendency to remove the pigment from the urine along with the protein; but sodium sul-

<sup>4</sup> Ville, J., and Derrien, E., *Compt. rend. Soc. biol.*, 1907, lxii, 679.

fate has little action on the pigments, and the protein when washed is pure white. The difficulty mentioned by Boggs and Guthrie<sup>5</sup> in obtaining a pigment-free precipitate is thus avoided.

If the urine at natural reaction is saturated with sodium chloride a slight opalescence results, but half saturation produces no effect. However, if sufficient acetic acid is added, precipitation is complete. Thus:

Urine.....	100 parts	} Gradual and incomplete precipitation.
50 per cent acetic acid . . .	S "	
Saturated solution NaCl . . .	10S "	
Urine.....	100 "	} Prompt and complete precipitation.
50 per cent acetic acid . . .	20 "	
Saturated solution NaCl . . .	120 "	

When the clear filtrate from this last precipitation is added to an equal volume of Bence-Jones urine only a slight precipitate is caused; but if more acetic acid is added heavy precipitation occurs at once. It would seem that a large amount of acid is fixed in breaking up some alkaline combination in which the protein is normally found, and that most salts precipitate it quantitatively only after this decomposition has been effected.

Spiegler's solution, Roberts' reagent, and acetic acid and ferrocyanide all cause immediate heavy precipitation when used with the native urine; and all are capable of indicating minute traces of this protein. This is of course what would be expected, and is mentioned only because of the fact found by Folin and Denis<sup>6</sup> that the Bence-Jones protein gave no precipitate with acetic acid and ferrocyanide except after long standing.

*Acids.*—Concentrated sulfuric, nitric, and hydrochloric acids added drop by drop cause a heavy white curdy precipitate, insoluble in moderate excess of the acid. A large excess causes solution by destruction, which is indicated by the change of color in the case of the sulfuric and nitric acids. Acetic acid added drop by drop may at first cause a very slight cloudiness (often entirely absent—nucleoprotein) which disappears upon adding a little more acid. Citric acid causes no cloudiness.

<sup>5</sup> Boggs, T. R., and Guthrie, C. G., *Am. J. Med. Sc.*, 1912, cxliv, 803.

<sup>6</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xviii, 277.

Urine diluted with two parts of water and treated with a small amount of either of the three mineral acids mentioned gives a dense white precipitate which dissolves upon boiling and reprecipitates upon cooling.

This solvent power of hot acids furnishes a satisfactory means of distinguishing between normal serum- and Bence-Jones protein. To a test-tube containing a few cc. of the urine to be examined is added a drop of concentrated nitric acid. This produces a white cloud or white curdy precipitate according to the amount of protein present; if a heavy curd is formed the urine should be diluted with water. Upon shaking, the precipitate disappears. Continue adding the acid drop by drop, shaking between each addition, until the precipitate or cloud no longer dissolves, then add one or two drops more. The contents of the tube are now brought to boiling. If the precipitate is Bence-Jones protein it will quickly dissolve, and upon cooling reprecipitates with the same white appearance as before; and this solution and precipitation can be repeated. If, on the other hand, the precipitate is due to normal serum protein it does not dissolve on boiling, but assumes the form of discrete flocks of a dirty yellowish or brownish color, not affected by cooling and not resembling in any way the white Bence-Jones precipitate.

Precipitation with HCl has been recommended as a specific test for Bence-Jones protein in the urine by Bradshaw<sup>7</sup> who states that albumin does not give this reaction unless present in very large amounts. Using strong HCl he assigns the limit of sensitiveness to Bence-Jones protein as 0.05 per cent, but apparently did not ascertain the percentage of serum protein that can be present without reacting. By a number of trials we found that the best differentiation occurs when: (1) the percentage of proteins is small; (2) the acid is comparatively weak, about 1 to 20 or 25; (3) when dilute acid is used, because if strong acid is added there is likely to be an immediate, more or less irreversible precipitation with either protein at the point of contact; (4) the mixture of urine and acid must be allowed to stand for a definite time, since eventually either protein will come down more or less completely.

<sup>7</sup> Bradshaw, T. R., *Brit. Med. J.*, 1906, ii, 1442.

In accordance with these principles the following method of testing was adopted. The dilute hydrochloric acid used consisted of concentrated acid one part, water three parts. In making the test the tube was well shaken after each addition, and after the acid was added was allowed to stand 1 minute before observing.

Normal urine.....	5	} No reaction.
Normal dog serum.....	0.25	
HCl (diluted).....	1	
Normal urine.....	5	} No reaction or doubtful.
Serum.....	0.5	
HCl (diluted).....	1	
Normal urine.....	5	} Doubtful or very faint.
Serum.....	1	
HCl (diluted).....	1	
Normal urine.....	5	} Very faint opalescence.
Serum.....	2	
HCl (diluted).....	1	

Even the last test was so faint that it would probably be rejected; but calling it positive, and assuming 6.5 per cent as the total protein content of the dog serum, the least amount of serum protein which will react with this test is 0.13 gm. protein or 1.86 per cent.

Testing now with Bence-Jones protein:

Normal urine... ..	5	} Faint opalescence.
Bence-Jones urine (2.7 per cent) ..	0.25	
HCl (diluted).....	1	
Normal urine....	5	} Well marked opalescence.
Bence-Jones urine (2.7 per cent) ..	0.5	
HCl (diluted).....	1	

The first of these two tests is somewhat clearer than the last of the previous series. However, to be on the safe side, if the second test be taken as the limit then the content is 0.013 gm. protein; thus, urine with 0.23 per cent Bence-Jones protein will react in this test. It is evident that this reaction is at least eight

times more sensitive for the latter than for the blood proteins. The ring test as described by Bradshaw although far more sensitive is less specific.

Besides the above hydrochloric acid test, we have found that lead acetate and zinc sulfate each have in the presence of acetic acid and under certain conditions power of differential precipitation between Bence-Jones protein and serum proteins. However, this action seems to depend upon so many factors that we have not so far succeeded in working out a practicable method of using these salts to distinguish between the proteins.

From a review of these reactions, especially the conduct with heat, where there is nearly always failure to obtain a quite complete re-solution upon boiling, we are inclined to believe that associated with the Bence-Jones protein is a small amount of serum protein; this conclusion is supported by the fact that a few casts are usually to be found upon centrifugation.

*Separation of the Protein.*—To each 100 cc. of filtered urine are added 20 cc. of 50 per cent acetic acid, and 120 cc. of a twice cold saturated and still slightly warm solution of sodium sulfate. This is stirred, allowed to stand about 20 minutes, and filtered with suction, using hardened paper. When the surface is dry the precipitate is washed three times with a cold saturated solution of sodium sulfate well acidulated with acetic acid. As soon as dry from the last wash solution, but while still pasty, it is scraped off from the paper and rubbed up in a dish to a thin milky suspension with 95 per cent alcohol. This rubbing is lightly done and the suspension is kept very fluid so as to leave behind as much of the sulfate as possible. From time to time the milky fluid is poured off into centrifuge tubes, a fresh portion of alcohol added to the dish, and the rubbing continued, always pouring off and renewing the alcohol before the suspension becomes rich enough to give the suggestion of cream. In this way the protein is gradually removed from the dish, together with some sulfate. Toward the end of the process the contents of the dish will be found to feel distinctly gritty from the sulfate crystals, and the operation should then be interrupted and the remainder discarded. The tubes are then centrifuged, the supernatant liquid is poured off, the tubes are filled with absolute alcohol, thoroughly stirred, and again

centrifuged. This alcohol is poured off and the washing process repeated. Then the protein is washed in the same manner once with a mixture of equal parts of absolute alcohol and ether, then twice with ether; finally it is collected on a filter of hardened paper and allowed to dry. Upon slight rubbing this protein will crumble to a fine white mobile powder, non-hygroscopic, scarcely soluble in water, but readily soluble in water or Ringer's solution when these are made slightly alkaline by dilute NaOH. In order to remove the remaining sulfate the dry protein is suspended in a large proportion of slightly warm distilled water in centrifuge tubes, stirred for a few minutes, and centrifuged. The wash water is then decanted from the protein, the alkaline water or other solvent poured in, and solution effected by stirring. Before dissolving, the washing with distilled water may be repeated, but this we usually omitted; if it be desired to obtain a strictly salt-free product this method should be replaced by dialysis.

When weak acetic acid is slowly added to a dilute slightly alkaline solution of the protein, some precipitation occurs at neutrality, which, however, readily redissolves upon the addition of a little more acid.

In alkaline solution the protein (like the native urine made alkaline) does not give the regular heat reaction. A solution of the protein with a very slight excess of acetic acid gives the reactions already described for native urine except that it does not coagulate upon heating. This is due to the absence of salts, for if a little sodium chloride is added, or especially if one or two volumes of normal urine are added to one volume of the protein solution, the typical heat behavior is fully restored.<sup>8</sup> It is therefore safe to assume that no material denaturation has been caused by this method of preparation. This conclusion is fully confirmed by the biological results described later.

For the purpose of estimating the protein, the simple heat precipitation in centrifuge tubes as described by Folin and Denis<sup>6</sup> (but with the use of only a trace of acetic acid) is very convenient.

<sup>8</sup> For the purpose of demonstration the protein may simply be suspended in normal urine of acid reaction, which is then boiled; whereupon the suspended particles promptly go into solution, with, again, a reproduction of the native Bence-Jones urine.



*Preparing a Protein-Free Urine.*—For subsequent work we required, in addition to the protein, urine from the same subject from which the protein had been quantitatively removed. The filtrate from the sodium sulfate precipitation was not suitable because of the large content of acid and besides it often contains traces of protein. We were not successful in strictly quantitative removal of the protein by heat alone; even the method given by Magnus-Levy<sup>2</sup> always allowed enough protein to remain in the filtrate to react with the Spiegler, Roberts, and the ferrocyanide tests. The following gave a strictly protein-free filtrate.

A mixture of 50 cc. of Bence-Jones urine, 50 cc. of water, and 100 cc. of sodium sulfate solution (100 gm. of sodium sulfate crystals in 200 cc. of water) is heated in the water bath; as soon as coagulation begins it is stirred occasionally with the thermometer until the temperature reaches 96–98°. Then it is taken from the bath, four drops of concentrated HCl are added, and the mixture is stirred well. After standing a minute or so it is rapidly cooled by immersing the vessel in cold water until the temperature has been reduced to about 40°, and filtered through 589 paper. The clear filtrate gives no reaction with any of the above reagents.

We also found that the protein can be quantitatively removed without heating, by the use of salt and acid. 2 cc. of concentrated HCl in 100 cc. of H<sub>2</sub>O are saturated with sodium chloride. An equal volume of urine is added, and the mixture stirred for a minute, and filtered. The clear filtrate is entirely free from protein. This salt-acid solution is of course essentially the Roberts reagent for albumin.

*Anaphylaxis.*—This protein is capable of causing active anaphylaxis, as the following experiments show.

*Anaphylaxis Series I.*

*Sensitization.*—Nov. 27. Twelve guinea pigs were injected subcutaneously. Nos. 1 to 6 each received 1 cc. of Bence-Jones urine (0.027 gm. of protein). Nos. 7 to 12 each received 1 cc. of a 2 per cent solution of protein in Ringer's solution (0.020 gm. of protein).

Dec. 23. All were injected; the injection was into the jugular vein except for No. 2 which was injected directly into the heart.

Animal No.	Intoxication. Dec. 23 (26 days).	Results.
1	Diluted Bence-Jones urine, 0.012 gm. protein.	Moderate anaphylaxis with complete recovery at end of 15 min.
2	" urine, 0.003 gm. protein.	Very slight anaphylaxis with recovery in 6 min.
3	" " 0.001 " "	Slight anaphylaxis with recovery in 11 min.
4	1 cc. Ringer's solution, 0.002 gm. protein.	No symptoms.
5	4 cc. Ringer's solution, 0.008 gm. protein.	Very slight anaphylaxis with recovery in 5 min.
6	1 cc. urine, 0.024 gm. protein.	Same as No. 5.
7	1 cc. diluted urine, 0.004 gm. protein.	Marked anaphylaxis with death in 7 min.
8	1 cc. diluted urine, 0.002 gm. protein.	Marked anaphylaxis with death in 9 min.
9	1 cc. Ringer's solution, 0.002 gm. protein.	Marked anaphylaxis with death in 8 min.
10	*	
11	1 cc. Ringer's solution, 0.002 gm. protein.	Moderate anaphylaxis with recovery in 13 min.
12	2 cc. Ringer's solution, 0.004 gm. protein.	Marked anaphylaxis with death in 5 min.

\* Lost.

From Series I it will be seen that: (1) the native Bence-Jones urine is capable of provoking only slight sensitization, whether the intoxicant is more of the urine or whether it is isolated protein; (2) the isolated protein is capable of causing very much higher sensitization against both native urine and isolated protein; (3) the native urine will intoxicate a sensitized guinea pig; (4) the isolated protein will also do this. The intoxicating power of the

native urine and of the isolated protein is about the same, so obviously the protein as prepared by us has suffered no material denaturation.

An interesting fact developed by this series is the weak sensitizing action of the native urine. Apparently some factor exists which prevents the protein from exerting its normal power; yet does not interfere with the subsequent intoxication of an animal properly sensitized by isolated protein. The experiments described in Series II were undertaken to study this inhibiting factor.

### *Anaphylaxis Series II.*

*Sensitization.*—Jan. 12. Six guinea pigs were injected subcutaneously. Nos. 1, 2, and 3 each received 1 cc. of Bence-Jones urine (0.026 gm. of protein). This urine had previously been heated at 55° for 1 hour. Nos. 4, 5, and 6 each received 4 cc. of the following solution: 0.1 gm. of Bence-Jones protein dissolved in 20 cc. of urine from which the protein had been removed as described, and then made slightly alkaline. Nos. 4, 5, and 6 therefore each received 0.020 gm. of protein.

Feb. 4. All were injected into the jugular.

Animal No.	Intoxication. Feb. 4 (23 days).	Results.
1	1 cc. of diluted Bence-Jones urine, 0.001 gm. protein.	No symptoms.
2	1 cc. of undiluted urine, 0.025 gm. protein.	Well marked anaphylaxis with death in 10 min.
3	0.5 cc. urine, 0.013 gm. protein.	Moderate anaphylaxis with recovery in $\frac{1}{2}$ hr.
4	1 cc. diluted urine, 0.001 gm. protein.	Marked anaphylaxis with death in 5 min.
5	1 cc. diluted urine, 0.0005 gm. protein.	Same as No. 4.
6	1 cc. diluted urine, 0.00025 gm. protein.	Moderate anaphylaxis with recovery in $\frac{1}{2}$ hr.

From Series II it is clear that the inhibiting factor is thermostabile at 55°; for the guinea pigs sensitized with the urine which had been heated at this temperature<sup>9</sup> showed about the same sen-

<sup>9</sup> The urine became slightly opalescent from beginning precipitation of protein, and was not filtered.

sitiveness, allowing for the larger intoxicating doses, as did the animals of Series I which were sensitized with unheated urine.

On the other hand, it is clear that the power of sensitization of the isolated protein when dissolved in the deproteinized urine is even greater than when the same protein is dissolved in Ringer's solution. The removal of the protein from the urine was effected by the first (sulfate and heat) method, described on page 290, and was undoubtedly quantitative within the limits of the tests there mentioned; therefore the greater sensitization cannot be attributed to the presence of protein unaccounted for. Evidently the inhibiting factor is present neither in the isolated protein nor in this protein-free filtrate; it is apparently destroyed by chemical manipulation or by heating to near the boiling point. We are at present unable to make any further statement regarding this matter.

The marked anaphylactic activity of this protein confirms the belief that we are dealing with a higher protein of individual biologic stamp and not with a degradation stage in the hydrolysis of any body protein. \*

*Direct Toxicity.*—While it has generally been assumed that Bence-Jones protein is not directly toxic, there seems to be in the literature no definite experimental evidence bearing on this question. We therefore tried the effect of a large dose. A dog was etherized, and received into the femoral vein 2 gm. per kilo of body weight of protein dissolved in slightly alkaline Ringer's solution. The dog remained etherized for about 2 hours, and during this entire time the blood pressure taken by cannula from the carotid artery and the respirations from the trachea were recorded by the continuous paper kymograph. From the femoral artery blood was taken at short intervals to test its coagulability. In no direction was there the slightest indication of toxic action; and the normal clotting of the blood speaks strongly against the presence of proteoses, which would have been in sufficient concentration to interfere with coagulation. This is quite in accord with the anaphylactic activity already described.

*Digestibility of Bence-Jones Protein.*—This protein in the circulation of the patient is, like the plasma proteins, protected from digestion; but the isolated substance, evidently undenatured, is readily attacked by either pepsin or trypsin.

In one of two flasks, each containing a little less than 100 cc. of water, were placed 1 gm. of protein and 0.1 gm. of Gruebler trypsin; in the other flask 1 gm. of protein and 0.1 gm. of pepsin. The flasks were then brought to the proper respective reactions, made up to 100 cc. volume, a small quantity of toluene was added, well shaken, and placed in the incubator at 39°. After 48 hours in the incubator 25 cc. were withdrawn from each flask, 225 cc. of absolute alcohol added to each, and the whole was allowed to stand about an hour. The pepsin digestion gave at once strong opalescence followed by a flocculent precipitate, which weighed 0.042 gm. The filtrate from this precipitate was well diluted with water, boiled down to drive off the alcohol, and then gave a strong biuret reaction, showing that substantial digestion had occurred. The trypsin digestion was treated in the same way. Weight of precipitate, 0.031 gm. filtrate as above.

The flasks were returned to the incubator for another 48 hours, when 25 cc. portions were taken as before. The precipitate from each of these portions was found to be only 3 mg. less than after the first period, and the digestions were not carried any further. It would appear therefore that while nearly all the protein yields readily, a small fraction resists digestion with considerable tenacity.

As control a 1 per cent solution of protein was precipitated without any digestion; the filtrate did not give a biuret reaction; so that at least within the limits of this test all undigested protein is thrown down by alcohol. As further control another trypsin digestion was set up with the same proportions as before, but was boiled 15 minutes before placing in the incubator. After 48 hours the filtrate from the alcohol precipitate gave no biuret reaction.

If the Bence-Jones protein is to be regarded as foreign to the tissues of the subject, we should by the Abderhalden hypothesis expect that a special ferment would soon be aroused to destroy it in the body; and certainly there would seem to be nothing in the nature of the substance itself to resist such a fate. Yet instead of being digested large quantities of it are excreted in its native condition, apparently free from any hydrolysis; and this can continue indefinitely. Under such circumstances we must dismiss the idea of a protective ferment.

Abderhalden and Rostoski<sup>10</sup> produced by several injections of Bence-Jones protein in rabbits precipitins which reacted not only with this protein but also with human serum and the proteins prepared from it. Hopkins and Savory<sup>11</sup> did not obtain precipitins (which they explain as probably due to injecting insufficient amounts of protein), but they state that

<sup>10</sup> Abderhalden, E., and Rostoski, O., *Z. physiol. Chem.*, 1905, xlv, 125.

<sup>11</sup> Hopkins, T. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.

anti-human serum gave a slight but definite reaction with solutions of Bence-Jones protein. Boggs and Guthrie,<sup>2</sup> by injecting solutions of Bence-Jones protein, produced in rabbits weak precipitins which reacted both to this protein and to blood proteins. In this connection it is interesting to note that Magnus-Levy<sup>3</sup> states that the character of the excretion of his patient slowly changed, becoming much less soluble on boiling than in the early stages of the disease, and finally behaved like ordinary blood protein. Yet he was satisfied that it was really the same substance because by changing the physical conditions of the test, as by the addition of salts or urea, the solubility at 100° returned.

From every point of view it is clear that this substance in our case as in others is a higher protein, a human product closely related to the normal blood proteins.

Our next paper will consider more fully the protein in the body and its elimination.

We desire to thank Dr. R. M. Pearce for his assistance with the anaphylaxis experiments; also Drs. J. E. Sweet and M. M. Peet for performing the experiment concerning the direct toxicity of the protein.



# THE UREASE CONTENT OF CERTAIN BEANS, WITH SPECIAL REFERENCE TO THE JACK BEAN.\*

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Urease has been found extensively in various bacteria, fungi, and higher plants. Takeuchi's discovery of urease in the soy bean<sup>1</sup> was followed by an investigation for urease in a number of the higher plant seeds by Kiesel<sup>2</sup> and later by Zemplén.<sup>3</sup> Falk<sup>4</sup> found urease in castor beans. Annett<sup>5</sup> examined a number of different varieties of the soy bean, finding the urease content about the same, while he also examined several Indian seeds for the presence of this enzyme. He made the interesting observation that the sword bean contained much more urease than the soy bean. Recently Benjamin<sup>6</sup> has noted the occurrence of urease in various nodules of the legumes and other plant parts.

Annett in speaking of the sword bean designates it *Canavalia ensiformis*. This designation properly belongs to the jack bean, a native of the West Indies and adjacent mainland, while the sword bean indigenous to India is properly designated *Canavalia gladiata*. These two beans have been much confused, and it is not quite clear with which bean this author has worked.<sup>7</sup>

In an examination of certain beans<sup>8</sup> we have found that the

\* This paper was presented in abstract at the meeting of the American Society of Biological Chemists, December, 1915.

<sup>1</sup> Takeuchi, T., *J. College of Agric.*, Tokyo, 1909, i, 1.

<sup>2</sup> Kiesel, A., *Z. physiol. Chem.*, 1911, lxxv, 169.

<sup>3</sup> Zemplén, G., *Z. physiol. Chem.*, 1912, lxxix, 229.

<sup>4</sup> Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 292.

<sup>5</sup> Annett, H. E., *Biochem. J.*, 1914, viii, 449.

<sup>6</sup> Benjamin, M. S., *Proc. Roy. Soc., New South Wales*, 1915, xlix, 78.

<sup>7</sup> For a discussion of the jack and sword beans see Piper, C. V., *U. S. Dept. Agric., Bureau of Plant Industry, Circular 110*, 1913.

<sup>8</sup> We desire to thank Dr. Piper of the U. S. Dept. of Agriculture for kindly furnishing us with most of the beans used in this work.



jack bean contains a very much larger quantity of urease than the sword or soy beans, and in fact shows a greater enzymatic activity toward urea than any other source of the enzyme. The urease of this bean is also apparently as specific for urea as that of the soy bean.

*Test of Beans for Urease.*

The test for urease in the various beans was performed as follows: The beans were finely ground in a coffee mill, and one part of the powder was treated with ten parts of distilled water and allowed to stand for 1 hour with occasional agitation. The suspension was then centrifuged, and the clear or slightly opalescent fluid siphoned off. Two 5 cc. portions of each of these extracts were placed in two flasks of 200 cc. capacity. 10 cc. of a 1 per cent solution of urea were added to one of the flasks, the other serving as a control. The fluid in each flask was made up to 100 cc., toluene was added to prevent bacterial growth, and the flasks were tightly stoppered and allowed to remain at room temperature. At the end of 20 hours the solutions were titrated with 0.1 N hydrochloric acid and methyl orange. Table I shows the results obtained.

TABLE I.

Botanical name.	Common name.	0.1 N HCl.	
		Test solution.	Control solution.
		cc.	cc.
<i>Phaseolus calcaratus</i> .....	Rice bean.	1.10	1.00
“ <i>angularis</i> .....	Adsuki “	1.20	1.10
“ <i>aconitifolius</i> ....	Moth “	1.50	1.30
“ <i>mungo</i> .....	Mung “	1.05	1.00
“ <i>aureus</i> .....	Urd “	5.30	1.00
<i>Stizolabium deeringianum</i> ..	Early Florida velvet bean.	1.60	1.50
	Chinese velvet bean.	1.60	1.60
<i>Vigna sinensis</i> .....	Cow pea.	1.10	1.10
<i>Dolichos lablab</i> .....	Hyacinth bean.	1.30	1.20
<i>Lupinus albus</i> .....	White lupine.	29.50	1.30
<i>Dolichos biflorus</i> .....	Horse gram.	32.80	1.00
<i>Glycine hispida</i> .....	Soy bean.	33.00	1.80
<i>Canavalia gladiata</i> .....	Sword “	33.00	1.60
“ <i>ensiformis</i> .....	Jack “	33.00	1.50

Six of the varieties of beans tested contained urease in sufficient amount demonstrable by this method. Three of these, white lupine,<sup>3</sup> soy bean,<sup>1</sup> and sword bean<sup>6</sup> have previously been shown to contain urease and are included for purposes of comparison. The samples which were negative by this test may contain traces of urease which would be detected by a more delicate or prolonged examination.

### *Relative Urease Activity.*

The relative activity of the bean extracts containing urease was next tested. The extracts used throughout the work were always prepared as described above unless otherwise stated. 10 cc. of each extract were added to 50 cc. of 1 per cent urea solution, 5 cc. portions removed at various intervals, and the amount of decomposition was determined by titration. Table II gives the results expressed in cc. of 0.1 N hydrochloric acid. These figures are corrected for the alkalinity of the bean extracts.

TABLE II.

Time.	0.1 N HCl.					
	<i>Phaseolus aureus.</i>	<i>Lupinus albus.</i>	<i>Dolichos biflorus.</i>	<i>Glycine hispida.</i>	<i>Canavalia gladiata.</i>	<i>Canavalia ensiformis.</i>
hrs.	cc.	cc.	cc.	cc.	cc.	cc.
$\frac{1}{4}$						13.70
$\frac{1}{2}$	0.20	0.20	1.20	4.10	10.70	13.70
1		0.45	2.15	6.30	13.45	13.60
$1\frac{1}{2}$	0.30	0.65	2.90	7.80	13.50	13.60
$2\frac{1}{2}$	0.40	0.90	4.00	11.10	13.50	13.65
18	0.90	3.40	11.60	13.60	13.40	13.60

Assuming that the enzyme concentration multiplied by the time is a constant, as has been shown for soy bean urease,<sup>9</sup> we see that the jack bean contains over fifteen times, and the sword bean about five times as much urease as the soy bean, while the *Dolichos biflorus* contains about  $\frac{1}{6}$ , the white lupine about  $\frac{1}{16}$ , and

<sup>9</sup> Marshall, E. K., Jr., *J. Biol. Chem.*, 1914, xvii, 351. Van Slyke, D. D., and Cullen, G. E., *ibid.*, 1914, xix, 141.

the *Phaseolus aureus* about  $\frac{1}{350}$  as much urease as the soy bean. These figures, of course, are very rough but indicate the great variation in urease content of the beans.

Inasmuch as the jack bean contains so much more urease than any of the others which have received investigation, further studies on this bean were made. Tables III and IV show the relative activity of the jack, sword, and soy beans. Extracts were prepared as usual and suspensions of the bean powders were also used. The two methods gave the same results, which fact is in favor of the view that the urease of each is practically completely extracted by the method employed.

The question of complete extraction was also tested directly by making an extract in the usual way, and then a second for a much longer time after rapidly washing the residue once from adherent remains of the first extract. The second extracts showed very little activity. An attempt was made to precipitate a part of the proteins by adding one-tenth volume of 0.1 N HCl to the extracts and filtering. This does not decrease the activity of the soy bean extract very much and was previously used by one of us. But with the sword bean and the jack bean extracts considerable loss of activity results.

In Table III 5 cc. of the extracts were added to 50 cc. of a 1 per cent urea solution, and 5 cc. portions of the mixture titrated at varying time intervals. In Table IV the experiment was similar to that of Table III except that 2 cc. portions of the extracts were used instead of 5.

TABLE III.

Time.	0.1 N HCl.								
	Soy bean extract.			Sword bean extract.			Jack bean extract.		
	Original.	Centrifuged.	Acidified.	Original.	Centrifuged.	Acidified.	Original.	Centrifuged.	Acidified.
hrs.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
$\frac{3}{4}$	3.1	3.0	2.5	7.9	7.8	3.7	15.65	15.6	10.2
$1\frac{1}{2}$	5.2	5.3	4.1	12.1	12.0	5.4	15.7	15.6	15.3
$2\frac{1}{2}$	7.3	7.4	6.0	15.3	15.0	6.8	15.6	15.6	15.45

TABLE IV.

Time.	0.1 N HCl.					
	Soy bean extract.		Sword bean extract.		Jack bean extract.	
	Original.	Acidified and filtered.	Original.	Acidified and filtered.	Original.	Acidified and filtered.
hrs.	cc.	cc.	cc.	cc.	cc.	cc.
$\frac{1}{2}$	1.50	1.30	3.70	2.00	8.60	4.40
1	2.30	2.20	6.20	3.20	14.00	7.00
$1\frac{1}{2}$	3.10	3.10	8.20	4.10	15.70	8.00

The total amount of solids present in the extracts was determined by evaporating 10 cc. portions to dryness in weighed porcelain dishes, drying over sulfuric acid *in vacuo*, and weighing the residues. The averages of three determinations on different samples were, in mg. of solids per 1 cc.:

	mg.
Jack bean.....	26
Sword bean.....	24
Soy bean.....	41
Acidulated extract of soy bean.....	30

The jack bean extract contains less solid material than either the aqueous or acidulated extract of the soy bean.

#### *Specificity of Jack Bean Extract for Urea.*

The action of the extract was tested on a number of nitrogenous substances. *Ereptone* was used as representing a mixture of amino-acids and polypeptides which occur in the body. 0.200 gm. was dissolved in 25 cc. of water and 10 cc. of the extract were added. *Casein*: 0.050 gm. was treated with 15 cc. of water, and 10 cc. of the extract; 0.050 gm. of *hippuric acid* and 0.050 gm. of *leucylglycine* were each treated with 15 cc. of water and 5 cc. of the extract. 0.025 gm. of *phenylalanine uramino-acid* was dissolved in 10 cc. of water, and 10 cc. of extract were added. The solutions were all made slightly alkaline to sodium alizarin sulfonate with 0.1 N sodium hydroxide. An amino-acid determination by Van Slyke's method was made on 2 cc. portions of each solution at once, and again the next morning. Table V gives the results in amounts of nitrogen gas obtained.

TABLE V.

Substance.	Initial reading.	Temperature.	Pressure.	Final reading.	Temperature.	Pressure.	Time between initial and final readings.
	cc.	°C.	mm.	cc.	°C.	mm.	hrs.
Ereptone*.....	1.58	19.5	760	1.57	19.5	762	18
	1.55						
Casein.....	0.50	26.5	763	0.50	24	761	13
	0.48			0.59			
Hippuric acid.....	0.44	28	757	0.44	26	758	19
	0.43			0.42			
Leucylglycine.....	1.20	28.5	757	1.18	26	758	19
	1.18						
Phenylalanine uramino-acid.....	0.80	28	760	0.88	28	763	20
Jack bean extract alone....	1.05	28	760	0.90	28	763	18
				1.14			

\* Ammonia determinations on 5 cc. portions of the creptone solution gave 1.10 cc. 0.02 N ammonia immediately and 1.20 cc. 0.02 N 18 hours later.

Table VI gives the results obtained on the action of the jack bean extract upon several other nitrogenous compounds occurring in animal fluids. 0.050 gm. of each substance was treated with 15 cc. of water, 10 cc. of jack bean extract were added, and

TABLE VI.

	Solution.	Control.
	0.02 N HCl required.	
	cc.	cc.
Jack bean extract alone.....	0.20	
Uric acid.....	0.20	
Creatinine.....	0.30	
Creatine.....	0.70	0.90
Adenine.....	0.25	0.20
Guanine.....	0.40	0.44
Glucosamine.....	1.30	1.30
Alloxantine.....	0.80	0.90
Béttaine.....	0.25	
Tyrosine.....	0.80	1.00
Cystine.....	0.20	

the mixture was made just alkaline to sodium alizarin sulfonate. This solution was allowed to stand for 16 to 20 hours, and the ammonia determined by aeration. In cases where the amount of ammonia was greater than that contained in the control sample of 10 cc. of the extract, a second control was carried out by allowing the substance and 10 cc. of the extract to stand the same length of time and mixing just before aeration.

*Preparation of the Enzyme in Solid Form by the Use of Organic Solvents.*

The acetone method used by Van Slyke and Cullen<sup>10</sup> for the soy bean urecase as well as other precipitants such as alcohol, mixtures of alcohol with ether, benzene, or ligroin were found to yield a dry powder of about the same activity per unit of weight as the original extract. 10 cc. portions of the jack bean extract, prepared by digesting one part of ground beans with ten parts of water and centrifuging, were poured into 100 cc. of the precipitant used. The acetone and mixture of two volumes of ether and one of alcohol gave the better yields (about 0.4 gm.) and also preparations of slightly greater activity. Tables VII and VIII show the comparative activity compared to the jack bean extract. In every case 25 cc. of a 1 per cent urea solution were added to the powder dissolved in 2 cc. of water, and 5 cc. portions titrated at varying time intervals.

TABLE VII.

Precipitant.	Weight of dried precipitate.	Time.			
		½ hr.	1 hr.	1½ hrs.	2 hrs.
		0.1 N HCl required.			
	mg.	cc.	cc.	cc.	cc.
Acetone.....	10	3.85	6.05	7.70	8.90
2 volumes of ether + 1 volume of alcohol.....	45.9	9.60	14.35	15.35	15.35
1 volume of benzene + 1 volume of alcohol.....	45.5	8.08	11.65	14.00	15.25
Jack bean* extract.....	45.0	9.85	14.20	15.25	15.30

<sup>10</sup> Van Slyke and Cullen, *J. Biol. Chem.*, 1914, xix, 211.

TABLE VIII.

Precipitant.	Weight of dried precipitate.	Time.	
		$\frac{1}{2}$ hr.	1 hr.
		0.1 N HCl required.	
	mg.	cc.	cc.
Acetone.....	49.5	12.80	15.45
Alcohol.....	48.7	11.00	15.40
1 volume of ligroin + 1 volume of alcohol.....	48.0	7.60	10.50
2 volumes of ether + 1 volume of alcohol.....	46.3	12.10	15.45
Jack bean* extract.....	57.7	12.40	15.45

\* Jack bean extract was added in a volume sufficient to contain this much dried residue.

The urease of the jack bean should prove useful especially in procedures involving the rapid removal of urea from a solution in which but little contaminating foreign material must be added. The extract obtained by digestion with water and centrifuging or some solid preparation can, of course, be used for the estimation of urea in urine directly by titration,<sup>11</sup> and a much shorter time is required than with the use of soy bean extract where turbidity and masking of the end-point preclude the use of excessive amounts of the extract. The results in Table IX show that with human urine or animal urines diluted to contain 2.5 per cent or less urca, the conversion is complete in  $\frac{1}{2}$  hour at ordinary room temperature. The determinations were carried out using 5 cc. portions of urine, and 2 cc. of the jack bean extract. The results are expressed in cc. of 0.1 N acid required.

TABLE IX.

No.	Time.		
	$\frac{1}{2}$ hr.	1 hr.	20 hrs.
	0.1 N HCl.		
	cc.	cc.	cc.
1	36.00	35.90	35.90
2	39.30	39.00	38.90
3	41.70	41.60	41.40
4	28.60	28.45	28.60

<sup>11</sup> Marshall, *J. Biol. Chem.*, 1913, xiv, 283.

The extract can also be used for the removal of urea preparatory to estimating amino-acids in urine by Van Slyke's<sup>12</sup> method, instead of soy bean extract as suggested by this author. A smaller amount of extract can be used, and the same time, giving a smaller blank, or the same amount of extract will require much less time.

#### SUMMARY.

Several varieties of beans have been examined for urease, and its presence or absence noted. The jack bean (*Canavalia ensiformis*) contains about fifteen times as much urease as the soy bean, while the extract from the jack bean contains much less solid residue than that from the soy bean. The urease of the jack bean as far as investigated appears to be as specific for urea as that of the soy bean. Suggestions are made wherein the urease of the jack bean may prove useful in chemical procedures.

<sup>12</sup> Van Slyke, *J. Biol. Chem.*, 1913-14, xvi, 125.





## NUTRITION INVESTIGATIONS UPON COTTONSEED MEAL. I.

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Whether or not cottonseed meal can safely and advantageously be utilized for human consumption is a matter which up to this time has received little attention. Several metabolism experiments have been carried out to determine the coefficient of digestibility of cottonseed meal,<sup>1</sup> and rabbits and pigs have been used in studying the question of the toxicity of cottonseed meal and its extracts.<sup>2</sup> In considering the broad question of the nutritive value of cottonseed meal, which must necessarily include the study of its efficiency in promoting growth, development, and reproduction, conclusions based upon the results of a metabolism experiment which occupies but a few days, or deductions from the fatal results of feeding excessive amounts, or extracts from excessive amounts of cottonseed meal, cannot alone be accepted as conclusive.

In this paper we present a preliminary report on the efficiency of cottonseed meal as a food for promoting the growth, development, and reproduction of the albino rat. The general plan of the investigation was suggested by the work of Osborne and Mendel, and the technique employed follows the methods observed by the authors on their visit to the Connecticut Agricultural Experiment Station.<sup>3</sup>

<sup>1</sup> Fraps, G. S., *Texas Agric. Exp. Station, Bull. 128*, 1910. Rather, J. B., *Texas Agric. Exp. Station, Bull. 163*, 1913.

<sup>2</sup> Withers, W. A., and Ray, J. B., *J. Biol. Chem.*, 1913, xiv, 53. Withers, W. A., and Brewster, J. F., *J. Biol. Chem.*, 1913, xv, 161. Crawford, A. C., *J. Pharm. and Exp. Therap.*, 1909-10, i, 519.

<sup>3</sup> We wish to thank Dr. Osborne and Miss Ferry for their advice and help as to the best method of conducting this work.

The rats used in the experiment were bred in Texas from Massachusetts stock. Through a careful process of elimination a sturdy resistant stock has been obtained. During the summer of 1915 the excessive heat in Texas appeared to have a slight retarding influence upon the growth not only of the experimental but also of the control animals.

Our plan of investigation includes the consideration of the following points.

1. Can animals live and thrive on a diet of which cottonseed meal comprises 33 to 50 per cent?
2. Can animals live and thrive on a diet in which cottonseed meal furnishes the only source of protein?
3. What nutritive factors are lacking in cottonseed meal to make it an efficient food *per se*?
4. Can cottonseed meal be combined with any foodstuffs to make an efficient diet?
5. Does cottonseed meal contain a toxic substance; if so, how does it act?

This paper reports the data of the first year's experiments, whose value will be more definitely determined by the results obtained from a longer period of experimentation.

In these experiments the control animals have been fed either an ordinary mixed diet consisting of dog biscuit,<sup>4</sup> milk, grain, green vegetables, and occasionally meat, or the following diet, which was recommended to us by Osborne and Mendel.

	<i>per cent</i>
Whole milk powder <sup>5</sup> .....	60
Starch.....	12
Lard.....	28

Cottonseed meal or flour, supplemented by other substances, was used in all the experimental diets. In the percentages of the protein content of these diets, we have imitated the control whole milk powder diet which supplies 25 per cent protein. All experimental diets are made up in paste form with 28 to 30 per

<sup>4</sup> Special dog biscuit manufactured by Potter and Wrightington, after the formula of Dr. Castle, of Bussey Institute. This biscuit was recommended by Osborne and Mendel for feeding stock rats.

<sup>5</sup> Whole milk powder containing 27.5 per cent butter fat, manufactured by Merrell-Soule, Syracuse, New York.

cent fat. We have used<sup>1</sup> two cottonseed products, cottonseed meal and Allison cottonseed flour. The flour has been made by refining commercial cottonseed meal and contains from 50 to 58 per cent protein and about 12 per cent fat. It has been deprived of the greater part of the lint, hulls, and resin, by the heating, bolting, etc., of the Baumgarten process.<sup>6</sup> The ordinary commercial cottonseed meal has been passed through a twenty mesh sieve to remove the lint and hulls to a uniform degree. This meal contains more hulls, resin, and coloring matter than the bolted flour. Both of these forms have been employed to test any nutritive advantage, or difference in degree of possible toxicity.

In the experiments the following diets have been used.

	<i>per cent</i>		<i>per cent</i>
1. Cottonseed flour.....	50	2. Cottonseed flour.....	33
Starch.....	22	Whole wheat flour <sup>7</sup> .....	37
Lard.....	28	Lard.....	30
3A. Cottonseed flour.....	50	4. Cottonseed flour.....	70
Starch.....	22	Lard.....	30
Butter fat.....	12		
Lard.....	16		
5. Ether-extracted meal... 50		7. Cottonseed flour..... 50	
Starch..... 22		Protein-free milk <sup>8</sup> ..... 22	
Lard..... 28		Butter fat..... 12	
		Lard..... 16	

<sup>6</sup> Manufactured by G. A. Baumgarten, Schulenburg Oil Mills, Schulenburg, Texas.

<sup>7</sup> Flour made from the whole crushed wheat, ground in the mill twice, and sifted through a twenty mesh sieve.

<sup>8</sup> For a short period protein-free milk was prepared according to the method of Osborne and Mendel, *Carnegie Institution of Washington, Publication No. 156*, pt. ii, 1911. Later it was found more convenient to use Merrell-Soule skim milk powder instead of fresh milk. After neutralizing the clear filtrate, the final step in the process described by these investigators, the liquid was again filtered to remove the small amount of protein precipitated by the neutralization. This preparation contains a smaller percentage of nitrogen than the original Osborne and Mendel preparations. These results were corroborated by the work of Mitchell, H. H., and Nelson, R. A., *J. Biol. Chem.*, 1915, xxiii, 461.

# 310      Nutritive Value of Cottonseed Meal.\*I

	per cent		per cent
8.	Sifted cottonseed meal.. 50	13.	Cottonseed flour..... 45
	Starch..... 22		Whole milk powder..... 17
	Lard..... 28		Starch..... 10
			Lard..... 28
	14.	Cottonseed flour..... 50	
		Protein-free milk..... 22	
		Lard..... 28	

## *Do Animals Live and Thrive on Cottonseed Meal?*

At the present time animals are alive after eating cottonseed meal diets for 310 to 345 days. These diets contained from 33 to 50 per cent cottonseed flour. The oldest animals have been on Diets 1 and 7 containing 50 per cent cottonseed flour. Others have received 50 per cent cottonseed flour in Diet 3 A and 33 per cent in Diet 2. While the animals on these diets have manifested a slower rate of growth than the control animals, yet the fact that rats are still alive after 310 to 345 days, approximately one-third of the extreme span of a rat's life,<sup>9</sup> on a diet which contains 33 per cent or more of cottonseed meal, is extremely important and one to be referred to in connection with the possible toxicity of cottonseed meal.

## *Cottonseed Meal as the Sole Source of Protein.*

With cottonseed flour as the sole source of protein, rats are still alive after 340 days. On Diet 1, in which the flour furnished 25 per cent protein, there have been growth, maintenance, and well nourished appearance in several rats for a period of about 135 days. Several rats which were continued beyond 135 days on Diet 1 steadily lost in weight, became greatly emaciated, and died after 152 to 157 days. Others recovered when additions were made to the diets. Rat 49, an especially sturdy animal, upon indicating failure to grow or maintain body weight on Diet 1 after 210 days was changed to Diet 3 A, which differed from Diet 1 only in 12 per cent butter fat content. With this addition of butter fat, recovery was almost complete. After 38 days failure was again evident, but an immediate gain in weight occurred

<sup>9</sup> Donaldson, H. H., Boas Anniversary Volume, New York, 1906, 5.

when the animal was supplied with Diet 7, which contains protein-free milk in addition to butter fat. Rat 36 on Diet 1 grew steadily for 137 days. At this point failure appeared and the animal's weight dropped so rapidly that the immediate substitution of an efficient diet was imperative. On Diet 7, containing butter fat and protein-free milk, this animal has recovered rapidly and reached practically normal full size at the age of 315 days.

Rats on Diet 4 made up of 70 per cent cottonseed flour and 30 per cent lard, have grown normally for 90 days. From this point they have grown very slowly or have maintained body weight and good appearance for 70 days longer. This diet is deficient in carbohydrate, some of the necessary inorganic constituents,<sup>10</sup> and possibly other substances essential in a complete diet (Chart 4). On Diet 4, Rat 44, although his resistance to such an adverse diet was not as great as that of other rats, demonstrated in a remarkable manner that this diet is sufficient for growth and maintenance for a considerable time. This animal on Diet 4, after reaching the age of 70 days, weight 83 gm., became extremely emaciated and too weak to stand. The animal's fur was rough and stood on end; feet, tail, and ears were colorless and transparent; and the eyes were swollen shut. At this point, when death seemed imminent, protein-free milk and butter fat were supplied as in Diet 7. Recovery was immediate and complete, normal growth was initiated, and after 42 days on this diet the rat was returned to 70 per cent cottonseed meal, on which it continued to grow for 77 days, maintained body weight for 52 days, and died at the age of 263 days, a well nourished, fat, fine-furred individual.

By referring to Chart 1 it appears that rats on 70 per cent cottonseed flour and 30 per cent lard have grown better than

<sup>10</sup> Ash analysis of Allison cottonseed flour:

	<i>per cent</i>
Inorganic salts.....	5.50
SiO <sub>2</sub> .....	0.14
Cl.....	None.
SO <sub>3</sub> .....	0.06
P <sub>2</sub> O <sub>5</sub> .....	2.57
K <sub>2</sub> O.....	2.01
CaO.....	0.26
MgO.....	0.25
Na <sub>2</sub> O.....	None.

Rats 111 and 50 on 50 per cent cottonseed flour, 28 per cent lard, and 22 per cent starch. In every case in which the diet showed a deficiency, the rats were recovered (Chart 2) by the addition of some substance other than protein to the diet. The fact that cottonseed flour has furnished the only source of protein for rats during 340 days and that rats have grown for 90 days and maintained body weight for 70 days more on a diet containing 70 per cent cottonseed flour and 30 per cent lard, is evidence that the protein of cottonseed flour is an available protein and is sufficient for growth.

### *What Nutritive Factors Are Lacking in Cottonseed Meal?*

Rats which received 12 per cent butter fat in Diet 3 A have not grown at any greater rate than those on Diet 1, but growth has continued for a longer period, probably due to the growth-promoting factor contained in butter fat<sup>11</sup> (Chart 3). With Rats 37 and 38 on Diet 3 A steady growth was maintained for 185 and 188 days before failure and, in the former case, death occurred. Rat 38 grew steadily for 197 days and maintained body weight for 39 days more; at this point the growth was resumed after improving the mineral content of the diet<sup>12</sup> by the addition of protein-free milk.

The recovery of Rats 36 and 49 on Diet 7 has already been mentioned. Rat 45 was reduced to a degree of emaciation and a condition similar to that of Rat 44 on the same Diet 4. When protein-free milk and butter fat were added as in Diet 7, a sudden growth impulse was manifested which persisted for 26 days when the animal died of lung trouble while still growing. With Rat 38 after 224 days of growth and maintenance on Diet 3 A, additional growth was promoted by 22 per cent protein-free milk. Rat 40 on Diet 1 grew slowly, probably due to a food intake below normal. Later with a more normal intake growth became pronounced. Eventually failure of the diet became evident, and when 12 per

<sup>11</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913-14, xvi, 423.

<sup>12</sup> McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615. Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Exp. Station, Research Bull.* 17, 1911. Osborne and Mendel, *J. Biol. Chem.*, 1913, 311.

cent butter fat was added, a slight but not continuous recovery occurred. However, when protein-free milk was added, a decided, immediate, and continuous growth was manifested (Chart 2).

We have not fed a diet containing ideal inorganic constituents, as protein-free milk and butter fat, for a long enough period to offer any conclusions as to the efficiency of such a diet over a long period compared with the whole milk powder control diet, or an ordinary mixed diet in promoting growth and development, but after 90 days on Diet 7, containing 50 per cent cottonseed flour, 22 per cent protein-free milk, 12 per cent butter fat, and 16 per cent lard, a large number of rats have grown normally and apparently are in as good physical condition as the control animals of the same age. This evidence considered together with the remarkable manner in which Diet 7 has recovered all rats reduced to extreme emaciation and possible death on incomplete diets, and induced immediate growth, indicates the lack in cottonseed meal of some essential nutritive factor rather than the presence of a deleterious substance. Whether this is due to deficiency in the inorganic salts<sup>10</sup> or to the lack of some growth-promoting factor has not been determined. The diet containing cottonseed flour and protein-free milk without butter fat has not been continued long enough to justify definite conclusions, but after 90 days on a diet containing 50 per cent cottonseed flour, 22 per cent protein-free milk, and 28 per cent lard, several rats have attained normal weight for control animals of the same age.

*Will Cottonseed Meal Combined with Some Common Food Material  
Make an Efficient Diet?*

In endeavoring to find a possible combination of cottonseed meal with some common food which would prove adequate for growth and development, cottonseed meal was combined with milk powder.<sup>12</sup> On this, Diet 13, perfectly normal growth and development of twelve animals have been obtained (Chart 5). Of the six females in this group, five have borne one or more litters of young. Rat 89 at the age of 146 days bore eight young, and ate all but four which were adopted by a stock female and after weaning were given Diet 13. At the present age of 70 days they

<sup>12</sup> Whole milk powder, Merrell-Soule.



are growing as well as control animals. 31 days after the first litter, Rat 89 bore five young. Rat 125 at the age of 148 days bore five young, and 56 days later, six young; Rat 134 at the age of 128 days bore seven young; Rat 136 at the age of 133 days bore six young, and 56 days later, five young; Rat 91 at the age of 189 days bore four young. The young rats after weaning have lived on the same Diet 13, as their mothers and are normal at the ages of 60 to 70 days.

This again points to the absence of an active toxic substance, since the addition of even 17 per cent of as favorable a food as milk powder could scarcely counteract the harmful effects of 45 per cent cottonseed meal if it contained an active toxic substance.

No young animals have been obtained from rats on Diets 1, 2, 3 A, 4, 5, and 8, containing from 33 to 70 per cent cottonseed meal or flour. All these diets were deficient in necessary minerals which alone might account for the inability to reproduce,<sup>14</sup> as it has been demonstrated that diets which are lacking in necessary inorganic constituents but are otherwise adequate are inefficient for the reproduction of the albino rat. We are at this time testing the possibility of reproduction on diets composed of cottonseed meal, butter fat, and essential inorganic salts furnished by protein-free milk, and we hope to report on this point later.<sup>15</sup>

### *Toxicity of Cottonseed Meal.*

Animals fed on cottonseed meal extracted with ether according to the method of Withers<sup>16</sup> have shown no nutritional advantages over those rats fed on the unextracted meal. In fact, failure and death occurred much sooner on the former diet, 5, than on the latter, 8. Perhaps this is due to the absence through extraction of the ether-soluble, growth-essential substance present in butter fat which appears to be present in small quantities in unextracted meal, since in the case of Rat 78, after a period of 240 days on a diet of ether-extracted meal, during which time there

<sup>14</sup> McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 615.

<sup>15</sup> Since this paper was started Rat 38, on Diet 7, has borne seven young, all in good condition.

<sup>16</sup> Withers, of the North Carolina Agricultural Experiment Station, believes that the ether extract of cottonseed meal contains a toxic substance which he calls *gossypol*.

was slight growth and later just maintenance, when butter fat was added to the diet pronounced growth was manifested.

In comparing the behavior of rats on cottonseed meal in Diet 8 and on the more highly milled flour in Diet 1, there is indication of a slight nutritive difference in the two products. Rat 86, Chart 1, on the ordinary meal, Diet 8, is still growing at a period beyond the time at which complete failure of Rat 50 on Diet 1 appeared. Chart 1 shows that while Rat 86 on Diet 8 has grown at a slower rate than Rats 50 or 111 on Diet 1, Rats 142 and 143, representative of animals of greater stamina, on Diet 8 are growing at a greater rate than any on Diet 1. This again suggests a greater amount of the growth-promoting substance associated with certain fats in the less highly milled product.

#### SUMMARY.

1. Albino rats are still alive after 310 to 345 days on diets containing 33 to 50 per cent cottonseed flour.

2. With 50 per cent cottonseed flour as the sole source of protein, mineral, and growth-promoting factors in Diet 1, rats have grown and maintained body weight for 135 days. With 50 per cent cottonseed flour as the sole source of protein rats have lived for 345 days. When cottonseed flour has been reinforced with protein-free milk to furnish adequate inorganic salts and butter fat containing a growth-promoting substance, rats which have failed on deficient diets have promptly recovered and exhibited a rate of growth nearly normal. Rat 38, thus recovered, has borne a litter of seven. Rats receiving 70 per cent cottonseed flour as the only source of protein, mineral, carbohydrate, and probable growth-promoting substances, have grown more normally for a period of 90 days, than the animals receiving 50 per cent cottonseed flour with the addition of 22 per cent carbohydrate as starch.

3. Rats receiving butter fat in a diet in which cottonseed flour is the only source of protein and mineral, show some nutritional advantage over rats not receiving butter fat in a similar diet, and have grown for a slightly longer period.

4. Rats which have failed on Diet 1 have recovered for a short period on Diet 3 A containing butter fat, but permanent recovery has occurred only when the inorganic salt content of the diet has been improved by the addition of protein-free milk as in Diet 7.

5. On a diet containing 45 per cent cottonseed flour, 17 per cent whole milk powder, 10 per cent starch, and 28 per cent lard, rats have grown and developed normally to the age of 6 months. Five females have borne one or more litters of young which are growing normally at the age of 60 to 70 days, on the same diet as the mothers.

6. Rats receiving cottonseed meal, containing more resin, fat, lint, and hulls than the flour, have shown nutritional advantage over those receiving cottonseed flour. This may be due to a greater amount of the growth-promoting factors in the meal than in the refined flour.

Our results indicate that cottonseed meal does not contain sufficient minerals for growth, is not actively toxic, contains efficient protein, and perhaps fat-soluble growth-promoting substances, similar to those of butter fat, but in less adequate quantities.

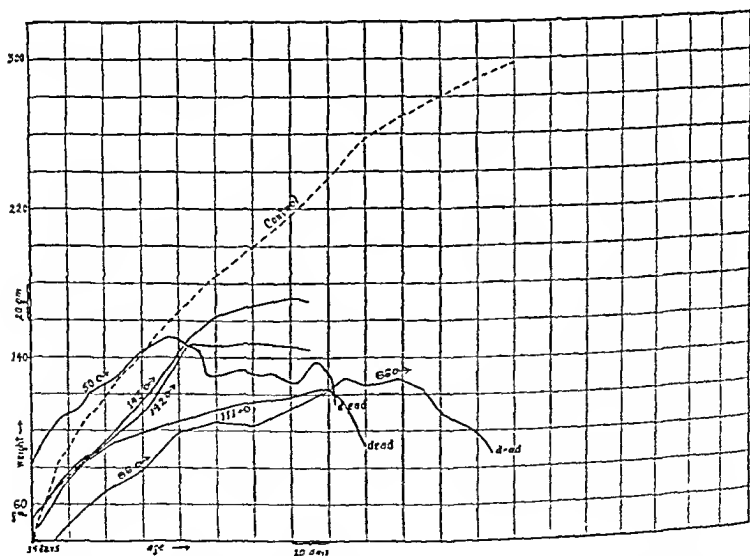


CHART 1. The growth of Rats 50 and 111 on refined cottonseed flour, Diet 1, and Rats 86, 142, and 143 on ordinary cottonseed meal, Diet 8. On the latter diet Rat 86 is growing at a period beyond the point at which rats, for example No. 50, have failed entirely on Diet 1. Rats 142 and 143, of sturdier stock than Rat 86, have grown almost normally for 110 days on Diet 8.

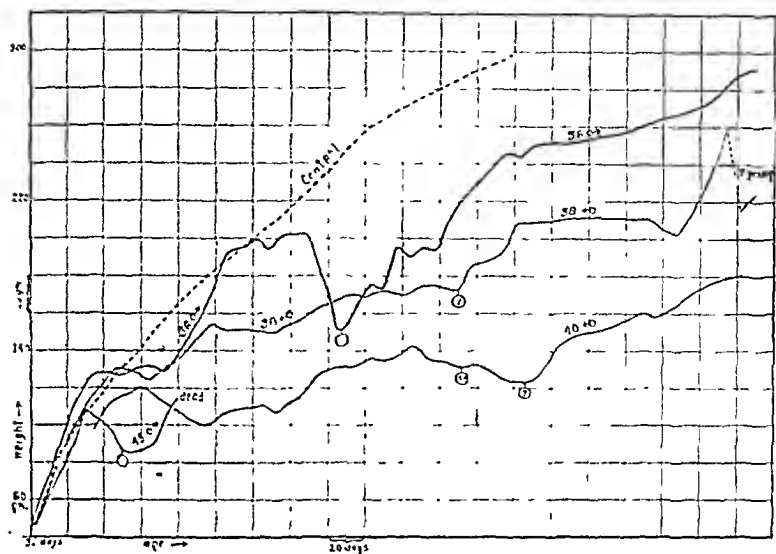


CHART 2. Rats which have failed on diets deficient in one or more essential nutritive factors have recovered on Diet 7, which supplies all of these factors. The point where figure 7 appears indicates when Diet 7 was given to each rat.

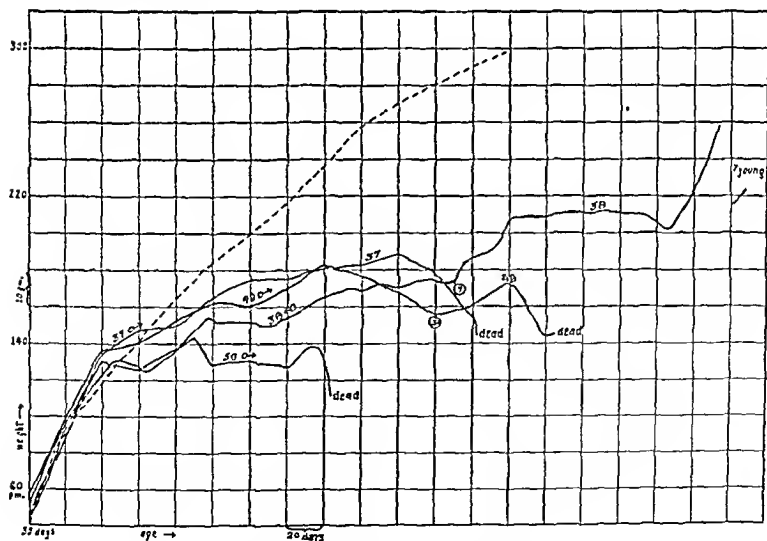


CHART 3. The efficiency of a cottonseed flour diet with butter fat, Diet 3 A, and without butter fat, Diet 1. Rats 38 and 37 on Diet 3 A continued to grow for 185 to 188 days, a longer period than Rats 49 and 50 on Diet 1 for 135 days. Note that Rat 38 was given further but not continuous growth by the addition of butter fat in Diet 3 A.

respond readily to the test. Proteins and their hydrolysis products were also found to give a positive reaction. One or two ammonium salts and a few bases were examined with negative results, and thus the test began to be regarded as characteristic of proteins and their hydrolysis products.

Herzfeld,<sup>5</sup> however, noticed that ammonium carbonate and ammonium oxalate when evaporated to dryness with triketohydrindene hydrate gave a positive reaction. It was, however, due to Neuberg<sup>6</sup> that the fact was discovered that the ninhydrin reaction with ammonium salts was a general one, thus contradicting the experiments and conclusions of Abderhalden and Schmidt. Neuberg also discovered the positive nature of the ninhydrin reaction with many organic bases and these results will be discussed in the succeeding paper.

The results of Neuberg destroy at once the specific nature of the ninhydrin reaction, and we wish in this paper to discuss the probable cause of the discrepancies and endeavor to gain some insight into the reaction.

*The Analogy between the Ninhydrin Reaction and the Formation of Murexide from Alloxan.*

This analogy was pointed out by Ruhemann,<sup>7</sup> who showed conclusively that the blue coloring matter produced by heating amino-acids and triketohydrindene hydrate was similar in properties and in method of preparation to murexide. Basing his ideas on the constitution of murexide advanced by Piloty and Finckh,<sup>8</sup> and by Slimmer and Stieglitz,<sup>9</sup> and the analogy between triketohydrindene hydrate and alloxan, he prepared the coloring matter in the following way.

Alloxan and triketohydrindene hydrate on reduction with mild reducing agents yield alloxantin and hydrindantin respectively, to which he gave the following constitutions.

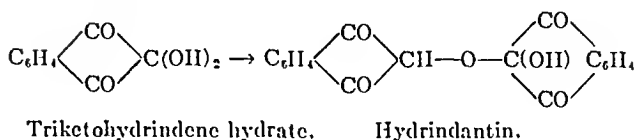
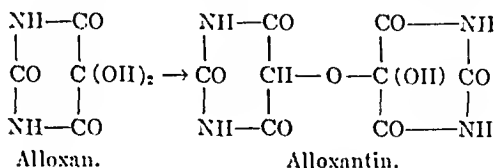
<sup>5</sup> Herzfeld, E., *Biochem. Z.*, 1914, lix, 249.

<sup>6</sup> Neuberg, C., *Biochem. Z.*, 1913, lvi, 500.

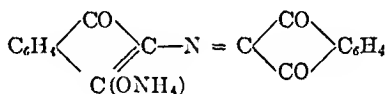
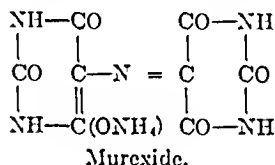
<sup>7</sup> Ruhemann, J. *Chem. Soc.*, 1911, xcix, 792, 1486.

<sup>8</sup> Piloty, O., and Finckh, K., *Ann. Chem.*, 1904, cccxxxiii, 22.

<sup>9</sup> Slimmer, M., and Stieglitz, J., *Am. Chem. J.*, 1904, xxxi, 661.



On warming with a mixture of ammonium carbonate and ammonium acetate these gave murexide and the ammonium salt of diketohydrindylidene-diketohydrindamine respectively.



Ammonium salt of diketohydrindylidene-diketohydrindamine.

The ammonium salt of diketohydrindylidene-diketohydrindamine prepared in this way through the intermediate hydrindantin was found to have a blue coloration identical with that formed by heating alanine and triketohydrindene hydrate, the alanine being oxidized to carbon dioxide and acetaldehyde. Triketohydrindene hydrate and glycine were shown to give formaldehyde, carbon dioxide, and the ammonium salt of diketohydrindylidene-diketohydrindamine. Strecker<sup>10</sup> had shown that alanine and alloxan, when heated together in aqueous solution, produced acetaldehyde, carbon dioxide, and murexide. Thus a close analogy between the two reactions was established.

The identity of the ammonium salt of diketohydrindylidene-diketohydrindamine was established by Ruhemann by means of analysis and chemical reactions. As a further means of identification, especially in dilute solution, we have observed three properties which can be used for the detection of the compound.

<sup>10</sup> Strecker, A., *Ann. Chem.*, 1862, cxxiii, 363.

a negative reaction is obtained in all cases. Thus when the concentration of the ammonium salt is such that 1.0 cc. = 0.05 mg. ammonium nitrogen, then, in all cases examined (twenty), with but one exception, a perfectly colorless solution resulted on heating them with 1.0 per cent aqueous ninhydrin solution (Table II). The one exception is ammonium sodium hydrogen phosphate, which gives a very faint trace of violet color under those conditions and the probable cause of this exception will be discussed later.

In the majority of cases we have been able to identify the blue or violet coloration as due, at any rate in part, to the ammonium salt of diketohydrindylidene-diketohydrindamine. We used the three tests mentioned on page 322, but in no case was sufficient color present to be determined quantitatively by the Harding-MacLean colorimetric method.<sup>15</sup> The discrepancies of previous observers can thus be accounted for; in concentration of about 1 per cent the ammonium salts of the weak organic acids give an unmistakable ninhydrin reaction, which is not the case when the concentration is extremely low (1.0 cc. = 0.05 mg. ammonium nitrogen).

In view of the results it became of interest and importance to determine whether ammonium salts in very dilute solution gave a ninhydrin reaction in the presence of pyridine. This modification of the ninhydrin reaction discovered by Harding and MacLean<sup>13</sup> results in a quantitative decomposition of the  $\alpha$ -amino nitrogen of amino-acids. How far, if at all, does it affect the decomposition of ammonium nitrogen when in similar concentration? The method of investigation was as follows:

Standard solutions of different ammonium salts were prepared of such a strength that 1 cc. of solution contained 0.05 mg. of ammonium nitrogen, thus being equivalent in strength to the standard alanine.

1 cc. of the ammonium salt solution together with 1 cc. of 10 per cent aqueous pyridine and 1 cc. of 1 per cent ninhydrin were heated for a period of 20 minutes in a rapidly boiling water bath. A strong blue color developed. The contents of the test-tube were cooled, diluted to 50 cc., and the amount of color was measured against a standard alanine color simultaneously prepared.

<sup>13</sup> Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1915, xx, 217.

For reasons given in a previous paper<sup>11</sup> the amount of decomposition of alanine, etc., is assumed to be quantitative. It will be seen by a glance at Table II that the decomposition of ammonium nitrogen under these conditions is constant and independent of the acid attacked, the two columns representing duplicate determinations.

TABLE II

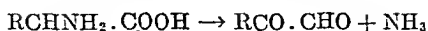
Ammonium salt.	Without pyridine	With pyridine Nitrogen decomposed	
		mg	mg
Chloride.....	No color.	0 019	0 020
Nitrate.....	" "	0 018	0 018
Sulfate .....	" "	0 017	0 016
Sodium hydrogen phosphate.	Very pale violet color.	0 016	0 015
Hydrogen phosphate ..	No color.	0 019	0 019
Carbonate.....	" "	0 018	0 018
Formate.....	" "	0 019	0 019
Acetate.. ....	" "	0 020	0 020
Oxalate... ..	" "	0 017	0 018
Succinate.....	" "	0 020	0 019
Glycollate.....	" "	0 019	0 019
Lactate.....	" "	0 019	0 019
Malate.....	" "	0 020	0 019
Tartrate.....	" "	0 018	0 019
Citrate .....	" "	0 015	0 015
$\beta$ -Oxybutyrate .....	" "	0 020	0 020
Benzoate.....	" "	0 020	0 022
Cinnamate ... ..	" "	0 020	0 020
Opianate.....	" "	0 018	0 018
Thiocyanate.....	" "	0 019	0 019

This decomposition of ammonium nitrogen is constant at about 0.018 mg. in 0.05 mg., or about 36 per cent, and the identification of the coloring matter as the ammonium salt of diketohydrindylidene-diketohydrindamine is readily effected by the use of the three afore mentioned tests.

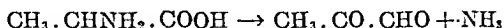
We next investigated the effect of varying amounts of ninhydrin and pyridine upon the reaction under conditions which parallel those in our studies on the decomposition of alanine. This is shown in Table III.

<sup>11</sup> Harding and MacLean, *J. Biol. Chem.*, 1916, **xiv**, 503.

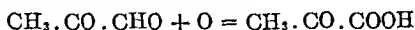




Their later work has inclined them to the belief that the reaction expressed is not a true chemical dissociation. In the case of alanine this would lead to the formation of methyl glyoxal and ammonia.



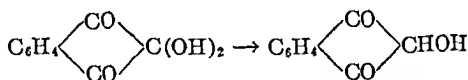
The glyoxals as a class are distinguished by being powerful reducing agents; they reduce ammoniacal silver oxide and Fehling's solution even in the cold. Thus it would be expected that a reduction of triketohydrindene hydrate would take place, giving rise to a substituted glyoxylic acid,



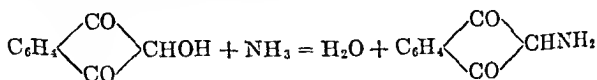
and that this would decompose into carbon dioxide and an aldehyde.



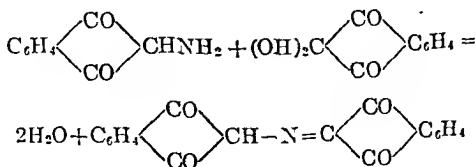
The triketohydrindene hydrate would be reduced to 1, 3-diketohydrindol



which on condensation with the ammonia from the amino-acid would give 1, 3-diketohydrindamine.

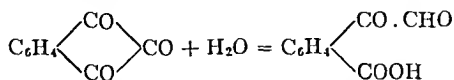


This compound readily condenses with aldehydes and ketones, is readily oxidized to a deep blue coloring matter,<sup>3,7</sup> and consequently would be expected to condense with a molecule of triketohydrindene hydrate to give diketohydrindylidene-diketohydrindamine, the ammonium salt of which is the required blue coloration.



Such a scheme accounts satisfactorily for the end-products of the reaction. It differs from that outlined by Ruhemann by not regarding hydrindantin as an intermediate product. The direct action of ammonia upon hydrindantin to produce diketohydrindylidene-diketohydrindamine would be expressed much better by a series of simple reactions involving the hydrolysis of hydrindantin to diketohydrindol and triketohydrindene hydrate. Moreover, we believe that we possess a certain amount of evidence against the view, for the presence of hydrindantin in large amounts reduces the amount of diketohydrindylidene-diketohydrindamine formed from an ammonium salt (page 333).

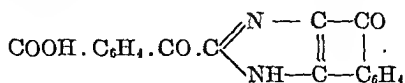
Again, as a working hypothesis, the intermediate formation of a glyoxal is attractive to us because it enables us to explain the ninhydrin reaction with ammonium salts. It is to be noticed that the ninhydrin reaction in the presence of pyridine is the same for all ammonium salts, being entirely independent of the acid attached; without pyridine it is most evident with the ammonium salts of the weak organic acids; *i.e.*, those salts which are the most readily hydrolyzed into their basic and acidic components, and with those salts which are faintly alkaline in reaction; *e.g.*, sodium ammonium hydrogen phosphate. Any reduction of the triketohydrindene hydrate under these circumstances must come from itself and the first action of the pyridine, or the weakly alkaline or dissociated salt must be the action of the hydroxyl ions upon the triketone. Ruhemann has shown conclusively that the first action of alkalis upon triketohydrindene hydrate is the formation of phenylglyoxal-*o*-carboxylic acid.



In other words, under the influence of weak alkalis the triketone itself can furnish the necessary glyoxal and the reaction would then proceed in the manner previously indicated. The formation of the yellow colored glyoxal can readily be observed on adding a few drops of 10 per cent aqueous pyridine to the triketone.

A necessary consequence of the hypothesis is that ammonia itself should give the ninhydrin reaction. Ruhemann investigated the action of ammonia upon the triketone and found

that he obtained a deep brownish red solution, from which by acidification he obtained a compound he regarded as 2-*o*-carboxy-benzoylindoglyoxaline,



The formation of the phenylglyoxal-*o*-carboxylic acid is evident, and this undergoes condensation with ammonia and a second molecule of triketohydrindene hydrate. We have found, however, that in very dilute solution the reaction with ammonia follows the reaction with ammonium salts. In a concentration of 1 cc. = 0.05 mg. ammonia nitrogen no reaction is obtained on heating 1 cc. with 1 cc. of 1 per cent ninhydrin for 20 minutes in a boiling water bath. In the presence of 1 cc. of 10 per cent aqueous pyridine, the ammonium salt of diketohydrindylidene-diketohydrindamine is formed and the decomposition of the ammonia nitrogen is 0.013 mg., a figure not far removed from the constant given by ammonium salts.

It is also easy to see why  $\beta$ - and  $\gamma$ -amino-acids have been found to give a faint ninhydrin reaction, and why acids with an amino group attached to a benzene ring fail to give the test, though we should expect that even here those aromatic amino-acids of which the amino group is readily hydrolyzed, would give a feeble positive reaction.

#### *The Action of Reducing Agents on the Ninhydrin Reaction with Ammonium Salts.*

It is evident that reducing reagents which assist the production of diketohydrindol from triketohydrindene hydrate should increase the amount of coloring matter formed with ammonium salts. And this in general we have found to be the case. We have examined a number of organic reducing agents which from their known reactions might be expected not to interfere with the condensations which take place, and measured their influence on the amount of coloring matter formed.

1 cc. of the standard solution of ammonium salt, 1 cc. of 10 per cent aqueous pyridine, 1 cc. of 2 per cent ninhydrin solution, and 1 cc. of the

reducing agent of the strength shown in Table IV were heated together in a boiling water bath for 20 minutes and the amount of nitrogen decomposed was estimated in the usual way. Control experiments on the reducing agent and ninhydrin in presence of pyridine gave negative results.

TABLE IV

Ammonium salt	Reducing agent	Concentration of reducing agent	N decomposed
		per cent	mg.
Chloride . . . . .	ethylene glycol.	10	0.025
" . . . . .	" "	100	0.035
" . . . . .	Glycerol	10	0.027
" . . . . .	"	100	0.032
" . . . . .	<i>p</i> -Hydroxybenzaldehyde.	1	0.029
" . . . . .	<i>p</i> -Hydroxybenzaldehyde.	3	0.032
" . . . . .	Glucose	0.5	0.025
" . . . . .	"	5.0	0.032
Nitrate . . . . .	"	0.5	0.027
" . . . . .	"	5.0	0.031
Sulfate . . . . .	"	0.5	0.026
" . . . . .	"	5.0	0.032
Acetate . . . . .	"	0.5	0.026
" . . . . .	"	5.0	0.032
Benzoate . . . . .	"	0.5	0.026
" . . . . .	"	5.0	0.032
Constant for ammonium salt..	1 cc. water.		0.025

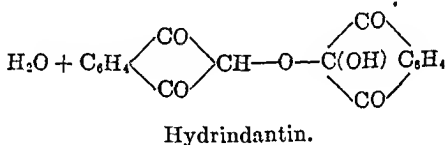
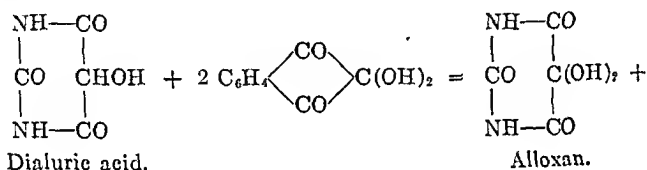
It will be seen that reducing agents have a distinct influence on the decomposition of ammonium nitrogen, though it is not so great as might have been expected, and in all cases a high concentration is necessary before the effect is shown. Thus a 0.5 per cent solution of glucose is without any influence and a 5 per cent solution only raises the decomposition from 50 to 64 per cent.

The results with ethylene glycol and glycerol are of interest, as they have a distinct bearing on the claims of Halle, Loewenstein, and Příbram<sup>16</sup> that these substances themselves give a ninhydrin reaction. They appear to act as very weak reducing

<sup>16</sup> Halle, W., Loewenstein, E., and Příbram, E., *Biochem. Z.*, 1913, *lv*, 357.

agents only, but this will be discussed more fully in another communication. The poor results obtained go to show that the reducing agent which takes part in the ninhydrin reaction must be either a very specific one or exceptionally powerful.

Of all the reducing agents we have examined dialuric acid is by far the most interesting. This acid was shown by Ruhemann<sup>17</sup> to give excellent yields of hydrindantin when heated with triketohydrindene hydrate, and we have repeated the observation several times in this laboratory; indeed, it is not necessary to heat the solutions, for in the concentrations at which we were working the hydrindantin rapidly crystallized out at the ordinary temperature.



A cold saturated solution of dialuric acid was prepared by dissolving the acid in hot water, cooling, and allowing the excess of acid to crystallize out over night (A). A second solution was prepared by dissolving dialuric acid in hot water, of such a strength that 1 cc. was capable of converting the whole of the triketohydrindene hydrate present in 1 cc. of a 2 per cent solution into hydrindantin according to the above equation (B). Such a solution of dialuric acid is supersaturated at ordinary temperatures, but as the dialuric acid does not crystallize out immediately, no difficulty is experienced in its use. Table V shows the effect of dialuric acid as a reducing agent on the ninhydrin reaction with ammonium salts, the experimental details being the same as those described on pages 330-331, and the figures representing mg. of ammonium nitrogen decomposed.

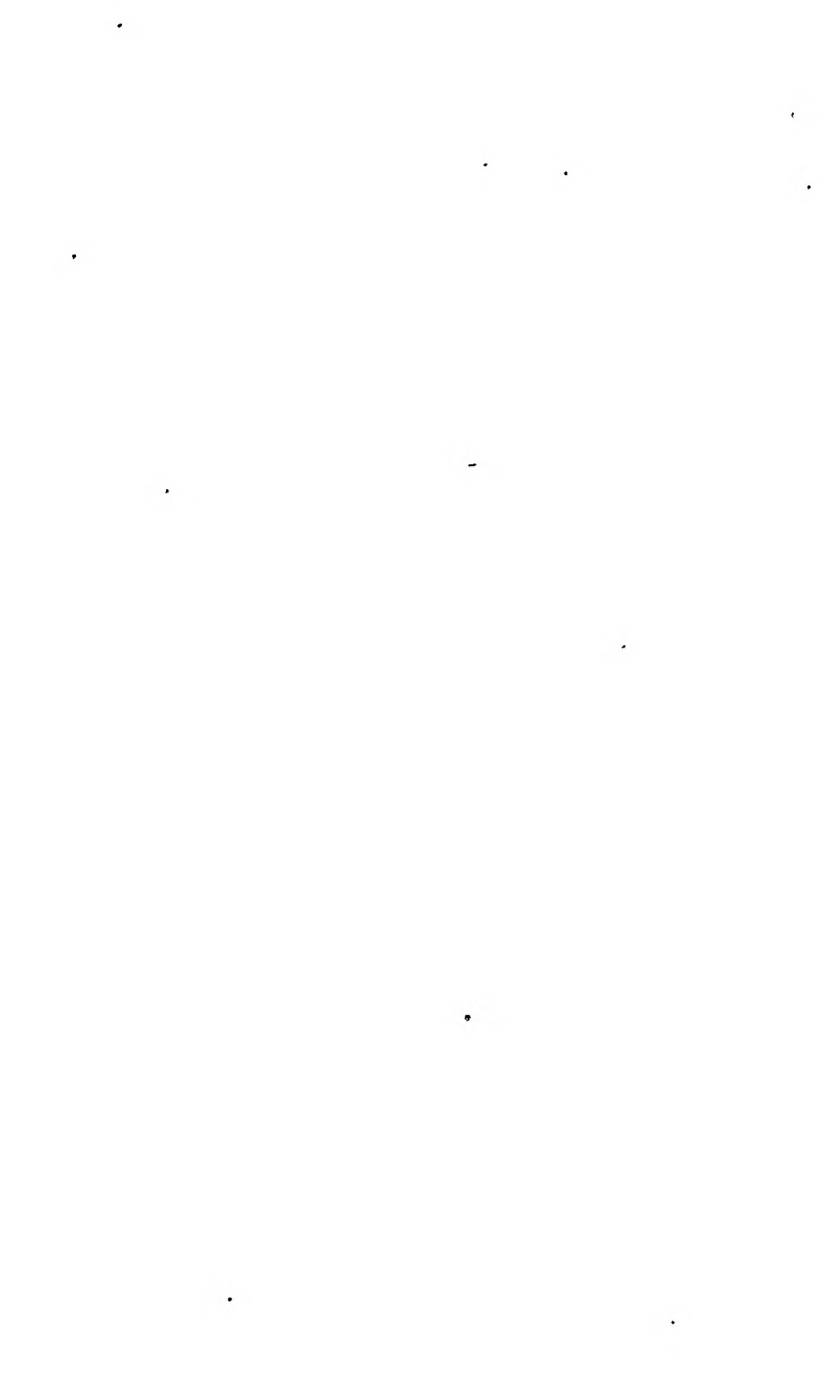
<sup>17</sup> Ruhemann, *J. Chem. Soc.*, 1911, xcix, 1310.

TABLE V

Ammonium salt.	+ 1 cc of water.	Dialuric acid	
		(A)	(B)
	mg	mg	mg
Chloride.....	0.025	0.015	0.031
Nitrate.....	0.025	0.014	0.031
Sulfate.....	0.026	0.013	0.031
Acetate.....	0.025	0.045	0.032
Benzoate.....	0.026	0.012	0.031

The striking influence of small amounts of dialuric acid is at once apparent. The cold saturated solution of the acid raises the decomposition of the ammonium nitrogen to nearly 90 per cent. The effect of the second solution of dialuric acid is not so marked. Here the solution contains a large excess of hydrindantin and its effect is not so great as that of smaller amounts; it is this fact that has induced in us the belief that hydrindantin probably does not form an intermediate step in the ninhydrin reaction. Moreover, the colors produced in the second series are of a very red shade, making the matching with the standard very difficult. In order to see if these red shades of color were due to the presence of alloxan or any derivative of it, or murexide, the hydrindantin formed by the interaction of the dialuric acid and the triketohydrindene hydrate was filtered off, washed on the filter with a little cold water, suspended in 2 cc. of distilled water, and a quantitative ninhydrin reaction was carried out with it. The solution obtained was extremely red in color and was found to contain only 0.013 mg. of decomposed nitrogen. A second experiment with pure crystallized hydrindantin gave an almost exactly similar result. The production of the reddish yellow colorations, then, is brought about by the excess of hydrindantin which does not decompose readily to give the ammonium salt of diketohydrindylidene-diketohydrindamine by the action of ammonia.

As the action of the cold saturated solution of dialuric acid had raised the decomposition of the ammonium salt to nearly 90 per cent, it became of interest and perhaps of practical importance to see if conditions could not be found under which the decomposition would become quantitative. Consequently a series of ex-



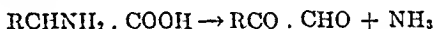
# THE NINHYDRIN REACTION WITH AMINES AND AMIDES.

BY VICTOR JOHN HARDING AND REGINALD M. MACLEAN.

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(Received for publication, April 22, 1916.)

In the preceding paper Harding and Warneford<sup>1</sup> have examined and discussed the interaction of amino-acids and ammonium salts with triketohydrindene hydrate (ninhydrin). They showed that the ninhydrin reaction with amino-acids could be explained by adopting the hypothesis of Dakin and Dudley<sup>2</sup> which supposes the decomposition of the amino-acid into ammonia and the corresponding glyoxal,



the glyoxal acting as the reducing agent. They showed that the ninhydrin reaction with ammonium salts, first pointed out as a general reaction by Neuberg,<sup>3</sup> only took place in the presence of hydroxyl ions, and that these had been observed by Ruhemann<sup>4</sup> to hydrolyze triketohydrindene hydrate to phenylglyoxal-*o*-carboxylic acid.<sup>5</sup>

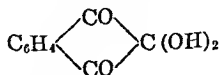
<sup>1</sup> Harding, V. J., and Warneford, F. H. S., *J. Biol. Chem.*, 1916, xxv, 319.

<sup>2</sup> Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 127.

<sup>3</sup> Neuberg, C., *Biochem. Z.*, 1913, lvi, 500.

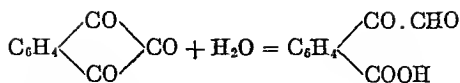
<sup>4</sup> Ruhemann, S., *J. Chem. Soc.* 1910, xcvii, 2026.

<sup>5</sup> In this and the preceding paper triketohydrindene hydrate has sometimes been written as the triketone for the sake of simplicity in explaining the decompositions. Its true formula, as pointed out by Ruhemann is,



and it will be found that we have used this where the reactions could be more clearly shown by its use.





In this way the requisite glyoxal was produced and the two series of facts were brought into agreement.

There still remains, however, the second criticism brought against the ninhydrin reaction by Neuberg,<sup>3</sup> who noticed that a large number of organic bases give a positive test with triketohydrindene hydrate. This is in contradiction to the statements of Abderhalden and Schmidt.<sup>6</sup>

In our experimental method we have followed the same procedure as that adopted in the previous paper. The bases examined were tested in varying concentration, with and without the presence of pyridine, and the general results followed those obtained with ammonium salts.

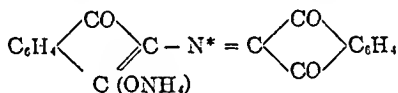
TABLE I.

Base	1 per cent solution	Solution 1 cc = 0.05 mg N		
		1 per cent ninhydrin	N decomposed in	
			1 per cent ninhydrin and pyridine	2 per cent ninhydrin and pyridine
			mg	mg
Methylamine	Strong reaction	No reaction	0.041	0.045
Ethylamine	" "	" "	0.037	0.044
Allylamine	" "	" "	0.021	0.022
Pentamethylenediamine	Reddish shades.	" "	+	+
Glucosamine	Strong reaction.	" "	0.043	0.046
Ethyl glycocholate	" "	" "	0.036	0.037
Phenocoll	" "	" "	0.021	0.021
Isopropylamine	No	" "	No reaction	
Aniline	" "	" "	"	"
Diethylamine	" "	" "	"	"
Diisopropylamine	" "	" "	"	"
ω-Aminoacetophenone	" "	" "	"	"

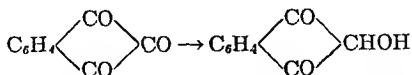
It is seen from an inspection of Table I that those bases which react with triketohydrindene hydrate do so in relatively high con-

<sup>6</sup> Abderhalden, E., and Schmidt, H., *Z. physiol. Chem.*, 1911, lxxii, 37.

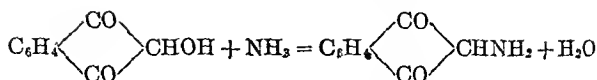
centration only. When in very dilute solution (1 cc. = 0.05 mg. amino nitrogen), no reaction is obtained. In the presence of pyridine, however, those bases which react in the higher concentration are found to react even in the very dilute solution. In this respect the ninhydrin reaction with amines follows that with ammonium salts. It is to be noted that if a base does not react with triketohydrindene hydrate alone when in the higher concentration neither does it do so when in the lower concentration and in the presence of pyridine. Furthermore, the amount of coloring matter (ammonium salt of diketohydrindylidene-diketohydrindamine) formed by the reactive bases in dilute solution in presence of pyridine is not constant, as it is in the case of the ammonium salts. The amount of color formed is distinctly and clearly dependent on the constitution of the amine from which it is formed, a point which we will discuss later. The identity of the coloring matter was proved by the three tests mentioned in the previous paper.<sup>1</sup> The constitution given to this compound,



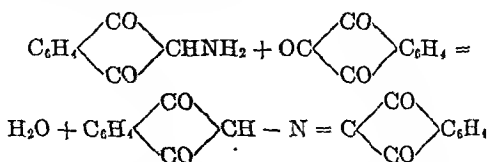
and its formation by the interaction of certain bases with triketohydrindene hydrate, mean a complete decomposition of the amines, in order to supply the nitrogen atom marked with the asterisk. The salt need not necessarily be an ammonium salt, as a salt with an organic base would fit the facts equally well, but the substitution of any other group for the nitrogen atom marked with the asterisk, or an alteration in the linkages, would cause a marked alteration in the absorption spectrum. In explaining the mechanism of the ninhydrin reaction with amino-acids, the nitrogen atom was supplied by the decomposition of the acid into the corresponding glyoxal and ammonia, the former causing a reduction of the triketone to 1, 3-diketohydrindol,



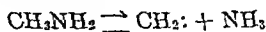
which condensed with loss of water with the ammonia.



A second molecule of triketohydrindene hydrate then condensed with the 1, 3-diketohydrindamine



to give diketohydrindylidene-diketohydrindamine. In the reaction with the ammonium salts the glyoxal was supplied by the hydrolysis of part of the triketone, the ammonia necessary to supply the nitrogen atom coming from the ammonium salt. The production of ammonia, however, from such organic amines as methylamine and ethylamine is a matter of difficulty. Such bases do not hydrolyze readily into the corresponding alcohol and ammonia. Any dissociation theory, such as that of methylamine into ammonia and methylene,<sup>7</sup>



has not sufficient facts in its favor to warrant its assumption. Moreover, such a dissociation theory would fail to explain the negative reaction with isopropylamine.

As the ninhydrin reaction with amines is clearly dependent on the constitution of the base, it becomes important to see if some insight into its mechanism cannot be gained by a classification of the bases, according to their action towards triketohydrindene hydrate and to their chemical constitution. This has been done and the results are given in the following tables.

In tabulating our own results we have always taken into consideration the concentration of the base, and a negative reaction given by us means that the base gives no blue coloring matter when 1 cc. of a 1 per cent solution is heated with 1 cc. of 1 per cent ninhydrin solution in a boiling water bath for a period of 20 minutes.

<sup>7</sup> Compare Hurtley, W. H., and Wooton, W. O., *J. Chem. Soc.*, 1911, xcix, 291.

TABLE II.  
 Type:  $RCH_2NH_2$ .

Base.	Observer.	Reaction.	Remarks.
Methylamine.....	{ Neuberg. Harding and MacLean.	+ +	
Ethylamine.....	{ Neuberg. Harding and MacLean.	+ +	
Isobutylamine.....	Neuberg.	+	Somewhat reddish.
Isoamylamine.....	"	+	
Hexylamine.....	"	+	Reddish.
Allylamine.....	{ " Harding and MacLean.	- +	Yellowish red. Reaction readily identified.
Benzylamine.....	Neuberg.	+	Reddish.
Phenylethylamine.....	"	+	
<i>p</i> -Hydroxyphenylethylamine.....	"	+	
$\beta$ -Iminazoethyamine.....	"	+	
$\beta$ -Indolethylamine.....	"	+	
Ethylenediamine.....	"	+	
Tetramethylenediamine.....	"	+	
Pentamethylenediamine.....	{ " Harding and MacLean. }	+	Reddish.
Aminoethylalcohol.....	Neuberg.	+	
Aminoacetal.....	"	+	
Aminoacetaldehyde....	"	+	
Ethyl glycocoll.....	{ Ruhemann. Harding and MacLean.	- +	
Phenocoll.....	" " "	+	
$\omega$ -Aminoacetophenone.	Neuberg.	+	
Taurine.....	"	+	In presence of sodium acetate.

The results given by this type of amine are clear and unequivocal. The only two exceptions previously known, *i.e.*, allylamine and ethyl glycocoll, on examination are shown to be errors. The finding with ethyl glycocoll ( $H_2NCH_2CO_2C_2H_5$ ) and phenocoll ( $C_2H_5OC_6H_4NHCOCH_2NH_2$ ) is of great interest as it finally dis-

poses of the claim of Ruhemann<sup>8</sup> and Abderhalden and Schmidt<sup>6</sup> that it is necessary for both the carboxyl and amino group of an amino-acid to be unsubstituted in order to obtain a positive result. The carboxyl group may be substituted, but substitution on the amino group certainly prevents the reaction (hippuric acid).

TABLE III.  
Type:  $R_2CHNH_2$ .

Base.	Observer.	Reaction.	Remarks
Isopropylamine.. . . .	Harding and MacLean.	—	Reddish.
Camphylamine.. . . .	Neuberg.	—	
Glucosamine.....	{ Abderhalden and Schmidt.	—	
	Neuberg.	+	Strong reaction.
	Harding and MacLean.	+	

The very small number of bases of this type which have been examined makes it difficult to draw definite conclusions. It would appear, however, that simple bases of this type do not react with triketohydrindene hydrate (isopropylamine and camphylamine). In the case of glucosamine, where we found an undoubted reaction, we have a negative aldehyde group on the carbon atom adjacent to the amino group, a fact which is well known to increase the reactivity of the neighboring groups, and the explanation of the reactivity of glucosamine may be sought in this direction. Moreover, it must not be forgotten that the amino-acids which are obtained by the hydrolysis of proteins and which show the greatest reactivity, belong to this group (except glycine). A number of di- and tri-peptides, quoted by Abderhalden and Schmidt as giving a positive reaction, are also to be classed in this category, so that the position occupied by glucosamine is not an isolated one. As a consequence we are brought to the conclusion that the grouping  $R_2CHNH_2$ , when one of the radicles is negative in character, reacts with ninhydrin, otherwise it is unreactive.

With the exception of the two tertiary amino-acids examined by Ruhemann, bases of this type do not give any reaction with ninhydrin. The two exceptions mentioned probably give a posi-

<sup>8</sup> Ruhemann, *J. Chem. Soc.*, 1911, xcix, 798.

TABLE IV.  
*Type: R<sub>3</sub>CNII<sub>2</sub>.*

Base.	Observer	Reaction
$\alpha$ -Aminoisobutyric acid.....	Ruhemann.	+
$\alpha$ -Aminoethylbutyric acid... .	"	+
$\beta$ -Aminoerotic ester... . . . .	Neuberg.	-
Adenine.....	"	-
Guanine . . . . .	{ Abderhalden and Schmidt.	-
	{ Harding and MacLean.	-
Aniline. ....	{ Neuberg.	-
	{ Harding and MacLean.	-
Benzidine.....	" " "	-
<i>p</i> -Aminoacetophenone ... . . . .	" " "	-

tive reaction on account of the ease with which tertiary amino-acids lose ammonia to form unsaturated acids. Indeed, Ruhemann<sup>8</sup> himself makes the observation that these two amino-acids required boiling with the reagent in order to give the reaction, whereas the other amino-acids only required warming.

TABLE V.  
*Types: R<sub>2</sub>NH and R<sub>3</sub>N.*

Base.	Observer.	Reaction
Diethylamine.....	{ Neuberg.	-
	{ Harding and MacLean.	-
Diisopropylamine. . . . .	" " "	-
Diisobutylamine. . . . .	" " "	-
Piperidine. ....	{ Neuberg.	-
	{ Harding and MacLean.	-
Adrenaline. ....	{ Neuberg.	+*
	{ Harding and MacLean.	+*
Indole... . . . .	Neuberg.	-
Skatole... . . . .	Harding and MacLean.	-
Trimethylamine .. . . .	" " "	-
Triethylamine....	{ Neuberg.	-
	{ Harding and MacLean.	-
Pyridine . . . . .	{ Neuberg.	-
	{ Harding and MacLean.	-
Quinoline . . . . .	{ Neuberg.	-
	{ Harding and MacLean.	-
Isoquinoline .. . . .	Neuberg.	-

\* Red shades.

With the exception of adrenaline secondary and tertiary bases are unreactive towards triketohydrindene hydrate. The instability of solutions of adrenaline is well known and this exception can well be explained in this way.

It is evident, then, that there are only two general classes of organic bases which give the ninhydrin reaction,  $RCH_2NH_2$  and  $R_2CHNH_2$  where one radicle is strongly negative in character.

In attempting to explain the ninhydrin reaction with these classes of organic bases, two facts must be borne in mind: (1) The reaction does not take place when the amine is in very dilute solution, unless in the presence of pyridine; *i.e.*, it parallels the reaction with ammonium salts. (2) The extent of the reaction depends on the constitution of the amine, the simpler amines being the more reactive unless a negative group is present (Table I).

In the previous paper we have shown how the analogy between triketohydrindene hydrate and alloxan enabled Ruhemann to arrive at the constitution of the blue color produced by the interaction of triketohydrindene hydrate and amino-acids. The analogy, however, between the formation of murexide from alloxan and its derivatives and the formation of the ammonium salt of diketohydrindylidene-diketohydrindamine in the ninhydrin reaction is much closer than has previously been recognized. In Tables VI and VII we have collected the results of the different investigators in the two fields. In Table VI we have collected the work of Ruhemann. The analogies, which were first drawn by him, are very clear and striking and extend not only to the methods of preparation of the compounds but to many of their physical and chemical properties. This is particularly striking in the case of the formation of chromo salts. It also shows the formation of 7-ethyluramil by the action of ethylamine on alloxantin, an observation which is as yet unknown in the hydrindene series. Table VII shows the relationships in the formation of murexide and the ammonium salt of diketohydrindylidene-diketohydrindamine. It shows that murexide can be formed by the action of ammonia, alanine (amino-acids), or ethylamine upon alloxan or alloxantin. If the analogy is a true one it is not surprising, then, to find that ammonia and ammonium salts, and organic bases of the type of ethylamine, in addition to amino-acids react with triketohydrindene hydrate to give the ninhydrin reaction.

*The Relation of Alloxan and Triketohydrindene Hydrate.*

Alloxan and derivatives.			Triketohydrindene hydrate and derivatives.		
Compound.	Preparation.	Formula.	Compound.	Preparation.	Formula.
Alloxan.		$\begin{array}{c} \text{NH} - \text{CO} \\   \\ \text{CO} - \text{C(OH)}_2 \\   \\ \text{NH} - \text{CO} \end{array}$	Triketohydrindene hydrate.		$\begin{array}{c} \text{CO} \quad \text{CO} \\ \diagdown \quad \diagup \\ \text{C(OH)}_2 \end{array}$
Violuric acid.	Hydroxylamine on alloxan. Nitrous acid on barbituric acid.	$\begin{array}{c} \text{NH} - \text{CO} \\   \\ \text{CO} - \text{C} = \text{NOH} \\   \\ \text{NH} - \text{CO} \end{array}$	Oxime 1,3-diketohydrindene.	Hydroxylamine on triketohydrindene. Nitrous acid on 1,3-diketohydrindene.	$\begin{array}{c} \text{CO} \quad \text{CO} \\ \diagdown \quad \diagup \\ \text{C} = \text{NOH} \end{array}$
Dialuric acid.	Reduction of alloxan by hydriodic acid.	$\begin{array}{c} \text{NH} - \text{CO} \\   \\ \text{CO} - \text{CHOH} \\   \\ \text{NH} - \text{CO} \end{array}$	1,3-Diketohydrindol.	Reduction of triketohydrindene hydrate by sodium amalgam.	$\begin{array}{c} \text{CO} \quad \text{CO} \\ \diagdown \quad \diagup \\ \text{CHOH} \end{array}$
Uramil.	Reduction of violuric acid. Action of ammonium chloride on hydrindantin.	$\begin{array}{c} \text{NH} - \text{CO} \\   \\ \text{CO} - \text{CHNH}_2 \\   \\ \text{NH} - \text{CO} \end{array}$	1,3-Diketohydrindamine.	Reduction of oxime 1,3-diketohydrindene.	$\begin{array}{c} \text{CO} \quad \text{CO} \\ \diagdown \quad \diagup \\ \text{CHNH}_2 \end{array}$
Alloxantin.	Reduction of alloxan by H <sub>2</sub> S.	$\begin{array}{c} \text{NH} - \text{CO} \\   \\ \text{CO} - \text{CH} - \text{O} - \text{C(OH)CO} \\   \\ \text{NH} - \text{CO} \end{array}$	Hydrindantin.	Reduction of triketohydrindene hydrate by H <sub>2</sub> S.	$\begin{array}{c} \text{CO} \quad \text{CO} \\ \diagdown \quad \diagup \\ \text{CH} - \text{O} - \text{C(OH)} \end{array} \begin{array}{c} \text{CO} \\   \\ \text{C}_6\text{H}_5 \end{array}$
7-Ethyluramil.	Action of ethylamine on alloxantin.	$\begin{array}{c} \text{NH} - \text{CO} \\   \\ \text{CO} - \text{CHNHC}_2\text{H}_5 \\   \\ \text{NH} - \text{CO} \end{array}$	1,3-Diketoeethylhydrindamine.	Unknown.	$\begin{array}{c} \text{CO} \quad \text{CO} \\ \diagdown \quad \diagup \\ \text{CHNHC}_2\text{H}_5 \end{array}$

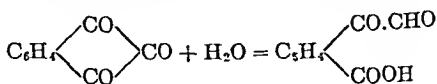


TABLE VII.

*The Relationship of Murexide and the Ammonium Salt of Diketohydrindylidene-diketohydrindamine.*

Murexide.		Ammonium salt of diketohydrindylidene-diketohydrindamine.	
Preparation from	By the action of	Preparation from	By the action of
Alloxan.	Ammonia in alcoholic solution.  Ethylamine. Amino-acids.	Triketohydrindene hydrate.	Ammonia in very dilute solution in presence of pyridine. Ethylamine. Amino-acids.
Alloxantin.	Ammonium acetate and carbonate. Ethylamine. Amino-acids.	Hydrindantin. -	Ammonium acetate and carbonate.  Amino-acids.
Uramil.	I <sub>2</sub> or HgO.  Alloxan in presence of ammonia.	1,3-Diketohydrindamine.	Air.
7-Ethyluramil.	Alloxan.		

The papers of Piloty and Finckh,<sup>9</sup> Slimmer and Stieglitz,<sup>10</sup> Möhlau,<sup>11</sup> and Möhlau and Litter<sup>12</sup> supply us with the clue to the ninhydrin reaction with amines. The similarity of the reaction with amines to that with ammonium salts in requiring the presence of large amounts of base or the presence of pyridine, again suggests that the production of phenylglyoxal-*o*-carboxylic acid represents the first stage in the mechanism



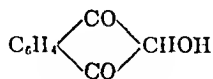
<sup>9</sup> Piloty, O., and Finckh, K., *Ann. Chem.*, 1904, cccxxxiii, 22.

<sup>10</sup> Slimmer, M., and Stieglitz, J., *Am. Chem. J.*, 1904, xxxi, 661.

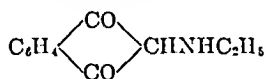
<sup>11</sup> Möhlau, R., *Ber. chem. Ges.*, 1904, xxxvii, 2686.

<sup>12</sup> Möhlau, R., and Litter, H., *J. prakt. Chem.*, 1906, lxxiii, 449.

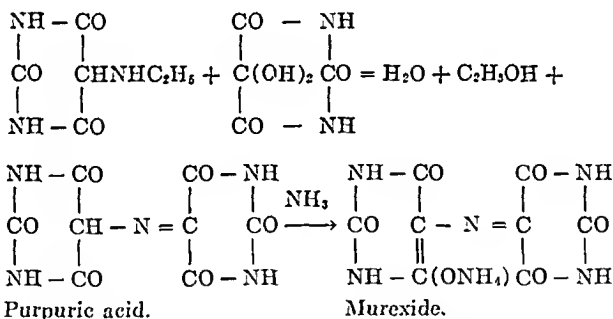
and that this acts as a reducing agent giving 1, 3-diketohydrindol or hydrindantin.



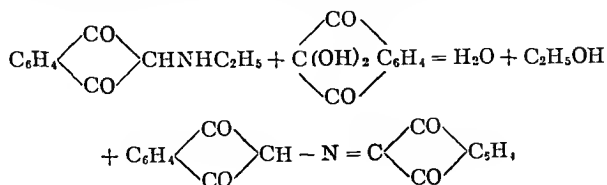
The action of ethylamine on alloxantin, as observed by Piloty and Finckh,<sup>9</sup> Slimmer and Stieglitz,<sup>10</sup> and Möhlau and Litter,<sup>12</sup> gives 7-ethyluramil, and there is no reason to suppose that the action of ethylamine on 1, 3-diketohydrindol or on hydrindantin would not produce 1, 3-diketoethylhydrindamine.



Just as 7-ethyluramil and alloxan in the presence of ammonia<sup>12</sup> give the ammonium salt of purpuric acid (murexide) and ethyl alcohol



so it would be expected that 1, 3-diketoethylhydrindamine and triketohydrindene hydrate would undergo a similar condensation to give diketohydrindylidene-diketohydrindamine



liquid with 1 cc. of 1 per cent ninhydrin solution in a boiling water bath for a period of 20 minutes, can be used as means of overcoming these difficulties. Even here, however, the solution must be free from large amounts of phosphates as these would tend to act like pyridine and, giving a few hydroxyl ions, cause a positive reaction with ammonium salts and certain amines.

These same criticisms apply with equal force to the quantitative reaction discovered by us.<sup>13</sup> It is only in the absence of large amounts of ammonium salts and bases that the method will yield accurate determinations of amino-acid  $\alpha$ -nitrogen, conditions which are fulfilled in the hydrolysis of proteins by pancreatic enzymes.<sup>14</sup>

The removal of ammonium salts and reactive amines, however, does not present insuperable difficulties, and it is hoped shortly to present a method of determining amino-acid  $\alpha$ -nitrogen in physiological fluids.

A large part of the experimental work described in this paper was carried out in the Biochemical Laboratory of Cornell University, Ithaca, N. Y., during the summer of 1915, and we wish to thank Professor S. Simpson and Dr. J. B. Sumner for the facilities they extended and for the many kindnesses received at their hands. Thanks are also due to Messrs. Parke, Davis and Company for the specimen of pure adrenaline.

#### SUMMARY.

1. The ninhydrin reaction is given by type  $RCH_2NH_2$ , and  $NH_2$  where  $R$  has the character.

2. Other bases oxidized give the

3. With the  $\alpha$  strongest reactive

4. Amides give

5. Guanidine "

daily yield reaction.

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<sup>13</sup> Harding, V. J.,

<sup>14</sup> Harding and M

<sup>15</sup> I., J.

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# THE INFLUENCE OF ALKALI AND ALKALINE EARTH SALTS UPON THE RATE OF SOLUTION OF CASEIN BY SODIUM HYDROXIDE.

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(Received for publication, May 4, 1916.)

One of the writers<sup>1</sup> has previously shown that when casein is shaken or stirred up in an alkaline solution, it dissolves rapidly at first, but later with slowness, and the relation between the time of stirring and the amount of casein dissolved obeys very accurately the relation  $x = Kt^m$ , where  $x$  is the number of grams of casein dissolved,  $t$  is the time, and  $K$  and  $m$  are constants which depend upon the concentration and kind of alkaline solution employed as solvent, and upon the total mass of casein present in the mixture. It appeared of interest to ascertain whether or not this relation held good in the presence of alkali or alkaline earth salts in the solvent, since these have a great influence upon the swelling and also upon the coagulation of colloidal substances, and, if the same relation exists when such solvent mixtures are employed, what influence these salts have upon the values of  $K$  and  $m$ . Accordingly the following experiments were undertaken.

The casein which was employed was Kahlbaum's casein *nach Hammersten*, specially purified by washing in distilled water, absolute alcohol, and ether according to the method described in a previous communication.<sup>2</sup>

The method of investigation which we adopted was that described in the previous paper;<sup>2</sup> that is, 100 cc. of the solvent were

<sup>1</sup> Robertson, T. B., *J. Phys. Chem.*, 1910, xiv, 377; *J. Biol. Chem.*, 1913, xiv, 237; *Arch. ges. Physiol.*, 1913, clii, 524.

<sup>2</sup> Robertson, *J. Phys. Chem.*, 1910, xiv, 377, 528

placed in a 400 cc. beaker of squat form and agitated by a flattened glass rod which was bent at right angles, the plane of the horizontal arm being somewhat inclined to the vertical, so as to communicate an upward thrust to the rotating liquid. The stirrer was rotated at an approximately constant rate by a small electric motor. While stirring, 5 gm. of casein were dropped into the fluid. At stated intervals samples of the mixture were almost instantaneously extracted by means of a 10 cc. pipette which was provided with a rubber bulb. The samples were then very rapidly filtered through lightly packed glass wool. Using a Pulfrich refractometer, the angle of total reflection (i) of the filtrate from each sample was measured and its refractive index thus determined. Denoting the refractive index of the filtrates from any given sample by  $n$  and that of the pure solvent by  $n_1$ , the quotient  $\frac{n-n_1}{0.00152}$  is the number of grams of casein<sup>6</sup> dissolved in 100 cc. of the solvent at the moment when the sample was extracted.<sup>3</sup>

For the standard solvent we used 0.016 N sodium hydroxide solution. The salts employed were lithium, potassium, and sodium chlorides in the concentrations of 0.5 N, N, and 2 N and calcium, strontium, and barium chlorides at the concentrations of 0.005 N, 0.01 N, and 0.05 N.

Though it was previously proved by one of the writers<sup>2</sup> that temperatures lying between 20° and 36°C. do not affect the rate of solution of casein in solutions of the hydroxides of the alkalies, in these experiments we maintained a fairly constant temperature, placing the stirring machine in a small double walled room warmed by an electric hot plate set at "Low." The temperature through the experiments lay between 22° and 24°C. The temperature during the progress of each experiment is given. In the column headed "Calculated" are given the values of  $x$  calculated from the above formula, the constants  $K$  and  $m$  being determined from the strongest results of the observations, employing for this purpose

4. Amides  $x = \log K + m \log t$ . The results obtained are
5. Guanidi

<sup>13</sup> Harding, V. J. *Phys. Chem.*, 1909, xiii, 469.

<sup>14</sup> Harding and

TABLE I.

t	i	n	C <sub>2</sub> sem in 100 cc of solvent		$\Delta$ (Difference)
			Observed.	Calculated.	

Solvent: 0.016 N NaOH.  $i = 68.05$ .  $n_1 = 1.329250$ . Temperature: 23.5 - 24.0°C.  $K = 2.371$ .  $m = 0.151$ .

min.			gm.	gm.	gm.
5	67.07	1.333711	2 98	3 02	+0 01
10	66 57	1.331497	3 45	3 36	-0.09
30	66.45	1.335415	4 08	3 96	-0.08
60	66.38	1 335990	4 43	4 40	-0 03
120	66.32	1.336170	4 75	4 91	+0 16
					$\Sigma\Delta = \pm 0 00$

Solvent: 0.016 N NaOH + 0.5 N LiCl.  $i = 66.45$ .  $n_1 = 1.335445$ . Temperature: 22.5 - 23.0°C.  $K = 1.640$ .  $m = 0.216$ .

5	66.01	1 338950	2 31	2 32	+0 01
10	65.53	1 339597	2 73	2 70	-0 03
30	65.40	1 340650	3 42	3 42	$\pm 0 00$
60	65 30	1 341470	3 96	3 97	+0 01
120	65.18	1 342454	4 61	4 61	-0 00
					$\Sigma\Delta = -0 01$

Solvent: 0.016 N NaOH + 1.0 N LiCl.  $i = 65.36$ .  $n_1 = 1.340778$ . Temperature: 22.5 - 23.0°C.  $K = 1.471$ .  $m = 0.223$ .

5	64.58	1 344106	2 19	2 11	-0 08
10	64.53	1 344521	2 46	2 47	+0 01
30	64.41	1 345526	3.12	3.14	+0 02
60	64.32	1 346274	3 62	3 67	+0 05
120	64 20	1 347280	4 28	4 28	$\pm 0 00$
					$\Sigma\Delta = \pm 0 00$

Solvent: 0.016 N NaOH + 2.0 N LiCl.  $i = 63.33$ .  $n_1 = 1.351292$ . Temperature: 22.5 - 23.0°C.  $K = 0.977$ .  $m = 0.223$ .

5	63.10	1 353270	1.30	1 40	+0 10
10	63 05	1 353705	1 59	1 63	+0 04
30	62 55	1 354575	2 16	2 09	-0 07
60	62 49	1 355097	2 50	2 43	-0 07
120	62.43	1 355619	2 85	2 84	-0 01
					$\Sigma\Delta = -0 01$

TABLE I—Continued.

<i>t</i>	<i>i</i>	<i>n</i>	Casein in 100 cc. of solvent.		$\Delta$ (Difference.)
			Observed.	Calculated.	

Solvent: 0.016 *N* NaOH + 0.5 *N* NaCl. *i* = 67.05. *n*<sub>1</sub> = 1.333870.Temperature: 22.0 – 23.0°C. *K* = 1.397. *m* = 0.225.

<i>min.</i>			<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
5	66.26	1.336950	2.03	2.01	-0.02
10	66.20	1.337430	2.34	2.35	+0.01
30	66.08	1.338390	2.97	3.00	+0.03
60	65.57	1.339273	3.55	3.51	-0.04
120	65.47	1.340083	4.09	4.10	+0.01
					$\Sigma\Delta = -0.01$

Solvent: 0.016 *N* NaOH + 1.0 *N* NaCl. *i* = 66.06. *n*<sub>1</sub> = 1.333559.Temperature: 22.0 – 23.0°C. *K* = 1.122. *m* = 0.228.

5	66.35	1.341060	1.65	1.62	-0.03
10	65.30	1.341470	1.92	1.90	-0.02
30	65.21	1.342208	2.41	2.44	+0.03
60	65.13	1.342864	2.84	2.85	+0.01
120	65.04	1.343608	3.33	3.34	+0.01
					$\Sigma\Delta = \pm 0.00$

Solvent: 0.016 *N* NaOH + 1.0 *N* KCl. *i* = 65.56. *n*<sub>1</sub> = 1.339354.Temperature: 22.5 – 23.0°C. *K* = 1.263. *m* = 0.221.

5	65.22	1.342126	1.82	1.80	-0.02
10	65.17	1.342536	2.09	2.10	+0.01
30	65.07	1.343359	2.63	2.68	+0.05
60	64.58	1.344106	3.13	3.12	-0.01
120	64.48	1.344938	3.67	3.64	-0.03
					$\Sigma\Delta = \pm 0.00$

Solvent: 0.016 *N* NaOH + 2.0 *N* KCl. *i* = 64.10. *n*<sub>1</sub> = 1.345130.Temperature: 22.5 – 23.0°C. *K* = 0.446. *m* = 0.243.

5	63.58	1.349150	0.67	0.66	-0.01
10	63.56	1.349320	0.78	0.78	$\pm 0.00$
30	63.52	1.349830	1.01	1.02	+0.01
60	63.49	1.349916	1.18	1.21	+0.03
120	63.44	1.350346	1.46	1.43	-0.03
					$\Sigma\Delta = \pm 0.00$

TABLE I—Continued.

<i>t</i>	<i>i</i>	<i>n</i>	Casein in 100 cc of solvent		$\Delta$ (Difference)
			Observed	Calculated	

Solvent: 0.016 *N* NaOH + 2.0 *N* NaCl *i* = 61.20.  $n_1 = 1.317280$ .Temperature: 22.0 – 23.0°C.  $K = 0.363$ .  $m = 0.270$ .

<i>min.</i>			<i>gm</i>	<i>gm.</i>	<i>gm.</i>
5	61.10	1.348130	0.56	0.56	$\pm 0.00$
10	61.08	1.348300	0.67	0.68	+0.01
30	61.04	1.348640	0.89	0.91	+0.02
60	61.00	1.348980	1.12	1.10	-0.02
120	63.56	1.349320	1.34	1.32	-0.02
					$\Sigma\Delta = -0.01$

Solvent: 0.016 *N* NaOH + 0.5 *N* KCl. *i* = 66.56.  $n_1 = 1.334576$ .Temperature: 22.5 – 23.0°C.  $K = 1.677$ .  $m = 0.207$ .

5	66.12	1.338070	2.30	2.34	+0.04
10	66.04	1.338710	2.72	2.70	-0.02
30	65.51	1.339759	3.41	3.39	-0.02
60	65.41	1.340569	3.94	3.91	-0.03
120	65.31	1.341388	4.48	4.52	+0.04
					$\Sigma\Delta = +0.01$

Solvent: 0.016 *N* NaOH + 0.005 *N* CaCl<sub>2</sub>. *i* = 67.59.  $n_1 = 1.329706$ Temperature: 23.0 – 23.5°C.  $K = 1.728$ .  $m = 0.197$ .

5	67.13	1.333246	2.33	2.37	+0.04
10	67.06	1.333792	2.69	2.72	+0.03
30	66.52	1.334892	3.41	3.38	-0.03
60	66.43	1.335603	3.88	3.87	-0.01
120	66.32	1.336470	4.45	4.44	-0.01
					$\Sigma\Delta = +0.02$

Solvent: 0.016 *N* NaOH + 0.01 *N* CaCl<sub>2</sub>. *i* = 67.58.  $n_1 = 1.329782$ Temperature: 22.0 – 23.0°C.  $K = 1.365$ .  $m = 0.213$ 

5	67.20	1.332700	1.92	1.92	$\pm 0.00$
10	67.15	1.333090	2.18	2.23	+0.05
30	67.03	1.334006	2.78	2.82	+0.04
60	66.52	1.334892	3.36	3.37	+0.01
120	66.42	1.335682	3.88	3.78	-0.10
					$\Sigma\Delta = \pm 0.00$



TABLE I—Continued.

<i>t</i>	<i>i</i>	<i>n</i>	Casein in 100 cc. of solvent.		$\Delta$ (Difference)
			Observed.	Calculated.	

Solvent: \* 0.016 N NaOH + 0.05 N CaCl<sub>2</sub>. *i* = 67.55. *n*<sub>1</sub> = 1.330010.  
 Temperature: 23.0 – 23.5°C. *K* = 0.459. *m* = 0.172.

min.			gm.	gm.	gm.
5	67.49	1.330467	0.60	0.60	±0.00
10	67.48	1.330544	0.70	0.68	-0.02
30	67.47	1.330660	0.85	0.82	-0.03
60	67.46	1.330698	0.91	0.93	+0.02
120	67.45	1.330775	1.01	1.05	+0.04
					$\Sigma\Delta = +0.01$

Solvent: 0.016 N NaOH + 0.005 N SrCl<sub>2</sub>. *i* = 67.56. *n*<sub>1</sub> = 1.329934.  
 Temperature: 22.5 – 23.0°C. *K* = 1.641. *m* = 0.200.

5	67.12	1.333324	2.23	2.27	+0.04
10	67.05	1.333870	2.59	2.60	+0.01
30	66.51	1.334971	3.31	3.24	-0.07
60	66.42	1.335682	3.78	3.72	-0.06
120	66.34	1.336310	4.19	4.27	+0.08
					$\Sigma\Delta = \pm 0.00$

Solvent: 0.016 N NaOH + 0.01 N SrCl<sub>2</sub>. *i* = 67.58. *n*<sub>1</sub> = 1.329782.  
 Temperature: 22.0 – 23.0°C. *K* = 1.135. *m* = 0.233.

5	67.24	1.332392	1.72	1.65	-0.07
10	67.19	1.332778	1.97	1.94	-0.03
30	67.09	1.333558	2.48	2.51	+0.03
60	67.01	1.334182	2.89	2.96	+0.07
120	66.50	1.335050	3.47	3.48	+0.01
					$\Sigma\Delta = +0.01$

Solvent: \* 0.016 N NaOH + 0.05 N SrCl<sub>2</sub>. *i* = 67.45. *n*<sub>1</sub> = 1.330774.  
 Temperature: 22.5 – 23.0°C. *K* = 0.462. *m* = 0.214.

5	67.39	1.331237	0.61	0.65	+0.04
10	67.37	1.331391	0.81	0.76	-0.05
30	67.35	1.331545	1.01	0.96	-0.05
60	67.34	1.331622	1.12	1.11	-0.01
120	67.33	1.331699	1.22	1.29	+0.07
					$\Sigma\Delta = \pm 0.00$

TABLE I—*Concluded.*

<i>t</i>	<i>i</i>	<i>n</i>	Casein in 100 cc of solvent		$\Delta$ (Difference)
			Observed	Calculated	

Solvent: 0.016 *N* NaOH + 0.005 *N* BaCl<sub>2</sub>. *i* = 67.58. *n*<sub>1</sub> = 1.329782.Temperature: 23.0 – 24.0°C. *K* = 1.660. *m* = 0.189.

<i>min.</i>			<i>gm.</i>	<i>gm.</i>	<i>gm</i>
5	67.14	1.333168	2.23	2.25	+0.02
10	67.07	1.333714	2.59	2.57	-0.02
30	66.55	1.334655	3.21	3.16	-0.05
60	66.47	1.335287	3.62	3.60	-0.02
120	66.39	1.335910	4.03	4.10	+0.07
					$\Sigma\Delta = \pm 0.00$

Solvent: 0.016 *N* NaOH + 0.01 *N* BaCl<sub>2</sub>. *i* = 67.59. *n*<sub>1</sub> = 1.329706.Temperature: 22.0 – 23.0°C. *K* = 0.842. *m* = 0.240.

5	67.34	1.331622	1.26	1.24	-0.02
10	67.31	1.331853	1.41	1.46	+0.05
30	67.21	1.332623	1.92	1.91	-0.01
60	67.14	1.333168	2.28	2.25	-0.03
120	67.07	1.333714	2.64	2.66	+0.02
					$\Sigma\Delta = +0.01$

Solvent:\* 0.016 *N* NaOH + 0.05 *N* BaCl<sub>2</sub>. *i* = 67.44. *n*<sub>1</sub> = 1.330852.Temperature: 23.0 – 24.0°C. *K* = 0.221. *m* = 0.243.

5	67.41	1.331083	0.30	0.33	+0.03
10	67.40	1.331160	0.41	0.39	-0.02
30	67.39	1.331237	0.51	0.51	$\pm 0.00$
60	67.38	1.331314	0.61	0.60	-0.01
120	67.37	1.331391	0.71	0.71	$\pm 0.00$
					$\Sigma\Delta = \pm 0.00$

\* Owing to the cloudiness of the solution obtained, it was diluted with the same volume of the solvent. The amount of the casein dissolved in 100 cc. solvent was, therefore, calculated according to the formula  $2 \times \frac{n - n_1}{0.00152}$ .

The possible experimental error in the determination of the concentration of a casein solution by means of its refractive index is always 0.07 gm. per 100 cc. It will be seen from Table I that the observed and calculated values of *x* are very close and

the differences ( $\Delta$ ) between these values are hardly ever greater, usually considerably less, than the possible error in the determination of the concentration of the casein in the filtrate. It is, therefore, evident that the relation between the time of stirring and the amount of casein dissolved obeys the relation  $x = Kt^m$  when alkali or alkaline earth salts are present in the sodium hydroxide solution employed as solvent. In each instance, however, the presence of the salt caused retardation of the rate of solution and this retardation (between the limits of concentration studied) was greater the greater the concentration of the salt.

In regard to their power of causing retardation of solution, there is a great difference between the salts of the alkalies and those of the alkaline earths. Alkali chlorides in 0.5 N concentration exert about the same retardation as alkaline earth chlorides in 0.005 N concentration. The power of the alkaline earth chlorides to retard the rate of solution of casein is, therefore, approximately 100 times greater than that of the chlorides of the alkalies. This remarkable quantitative disparity between the effects of alkaline earth chlorides and the chlorides of the alkalies is strikingly analogous to the familiar disparity of their effects upon living tissues. Now the fact that the law  $x = Kt^m$ , the time relationship which is characteristic of surface tension phenomena,<sup>4</sup> holds good in the presence of these salts shows that their effect upon the rate of solution of casein is primarily to be attributed to their effect upon the rate of penetration of the casein particles by the solvent. The possibility thus suggests itself that in the actions of salts upon living tissues we may also be dealing with the effects of alterations in penetrability, a view to which experiments of Osterhout<sup>5</sup> and Loeb and Beutner<sup>6</sup> afford substantial support.

The retarding effect of the salts employed increases in the following order:  $\text{Li} < \text{K} < \text{Na} < \text{Ca} < \text{Sr} < \text{Ba}$ .

From the fact that NaCl exerts only a slightly greater retarding influence than KCl although sodium hydroxide is the alkali

<sup>4</sup> Bell, J. M., and Cameron, F. K., *J. Phys. Chem.*, 1906, x, 658. Ostwald, W., and Goppelsroeder, F., *Z. kolloid. Chem., Suppl.*, 1908, ii, 29.

<sup>5</sup> Osterhout, W. J. V., *Science*, 1912, xxxv, 112.

<sup>6</sup> Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1.

employed as solvent, we may infer that the retarding effect of these salts cannot be wholly attributed to diminution of the dissociation of the hydroxide due to the presence of a salt with a common ion.

The magnitudes of the constants  $K$  and  $m$  are affected by the salts in opposite senses; and the value of  $K$  decreases as the concentration of salt increases, while that of  $m$  increases.

Differentiating the equation  $x = Kt^m$  we find:  $\frac{dx}{dt} = Kmt^{m-1}$  in other words, the product  $Km$ , which we may term the *coefficient of penetration* expresses the constant proportionality between the velocity of solution and an exponent (peculiar to each solvent) of the time during which the protein has been exposed to the action of the solvent.

Since the value of  $K$  decreases more rapidly than  $m$  increases with increasing concentration of salt, the effect of all the salts, within the limits of concentration employed, is to cause a decrease in the value of the coefficient of penetration, a decrease which is a definite function of the concentration of salt employed.

The relationship between the value of the coefficient of penetration and the concentration of salt added to the solvent may be defined by the interpolation equation  $K_1m_1 - Km = \alpha c + \beta$ ; where  $K_1m_1$  is the coefficient of penetration for the pure solvent (0.016 N NaOH),  $Km$  is its value when a given salt has been added in concentration  $c$ , and  $\alpha$  and  $\beta$  are constants dependent upon the nature of the added salt.

In Table II the observed values of the coefficient of penetration are compared with the values calculated from the equation, the values of the constants  $\alpha$  and  $\beta$  being calculated by the method of least squares from all of the observations obtained with a given salt.

These results reveal another striking difference between the effects of the chlorides of the alkalis and of the alkaline earths upon the rate of penetration of the casein particles in solvent, for whereas NaCl and KCl in increasing concentration decrease the rate of penetration with a *positive* acceleration,  $\text{CaCl}_2$ ,  $\text{SrCl}_2$ , and  $\text{BaCl}_2$  decrease the rate of penetration with a *negative* acceleration, which implies that at sufficiently high concentrations they would actually accelerate the penetra-

TABLE II.

Concentration.	Km		Difference.
	Found.	Calculated.	
LiCl. $K_1m_1 = 0.356$ . $\alpha = -0.013$ . $\beta = +0.041$ .			
0 00	0 356	0.356	$\pm 0.000$
0 50	0 354	0.352	$-0.002$
1 00	0.328	0.328	$\pm 0.000$
2 00	0.218	0.218	$\pm 0.000$
NaCl. $K_1m_1 = 0.356$ . $\alpha = +0.066$ . $\beta = +0.032$ .			
0 00	0.356	0.356	$\pm 0.000$
0 50	0.315	0.315	$\pm 0.000$
1 00	0.258	0.258	$\pm 0.000$
2 00	0.097	0.096	$-0.001$
KCl. $K_1m_1 = 0.356$ . $\alpha = +0.030$ . $\beta = +0.047$ .			
0 00	0.356	0.356	$\pm 0.000$
0 50	0.347	0.329	$-0.018$
1.00	0.279	0.279	$\pm 0.000$
2 00	0.108	0.108	$\pm 0.000$
CaCl <sub>2</sub> . $K_1m_1 = 0.356$ . $\alpha = +8.100$ . $\beta = -12.250$ .			
0 000	0.356	0.356	$\pm 0.000$
0 005	0.340	0.346	$+0.006$
0 010	0.291	0.289	$-0.002$
0 050	0.251	0.248	$-0.003$
SrCl <sub>2</sub> . $K_1m_1 = 0.356$ . $\alpha = +10.200$ . $\beta = -10.150$ .			
0.000	0.356	0.356	$\pm 0.000$
0.005	0.328	0.330	$+0.002$
0.010	0 264	0.264	$\pm 0.000$
0.050	0 099	0.099	$\pm 0.000$
BaCl <sub>2</sub> . $K_1m_1 = 0.356$ . $\alpha = +17.700$ . $\beta = -23.400$ .			
0.000	0.356	0.356	$\pm 0.000$
0.005	0.314	0.326	$+0.012$
0.010	0.202	0.202	$\pm 0.000$
0.050	0.054	0.054	$\pm 0.000$

the casein by the solvent. The study of the effects of higher concentrations of alkaline earth chlorides upon the rate of penetration of casein is, however, complicated by a number of factors which tend to obscure these relationships. Investigations of these effects are in progress and will form the subject of a subsequent communication. Lithium chloride occupies an exceptional position among the salts studied, since with increasing concentration it increases the rate of penetration with *negative* acceleration. This implies that at a sufficiently low concentration (less than  $\text{M}/3$ ) the presence of  $\text{LiCl}$  might be expected to accelerate the penetration of casein particles by  $0.016 \text{ N NaOH}$ . A simple calculation serves to show, however, that this effect even if present would be too small to be measurable by the technique employed.

#### CONCLUSIONS.

1. The relation between the time which has elapsed since the casein was introduced into the solvent and the amount of casein dissolved is expressed by the relation  $x = Kt^m$  even in the presence of alkali or alkaline earth chlorides in the sodium hydroxide solution employed as solvent.

2. The presence of these salts decreases the rate of solution of casein by dilute sodium hydroxide. The retardation increases with the concentration of salt employed.

3. The alkaline earth chlorides in  $0.005 \text{ N}$  concentration retard the rate of solution of casein approximately to the same extent as 100 times this concentration of a chloride of an alkali.

4. The value of  $K$  decreases while that of  $m$  increases with increase of the concentration of the salts. The value of the product  $K \times m$ , which we term the coefficient of penetration, decreases with increasing concentrations of  $\text{NaCl}$ ,  $\text{KCl}$ ,  $\text{CaCl}_2$ ,  $\text{SrCl}_2$ , or  $\text{BaCl}_2$  between the limits of concentration employed, the acceleration of the decrease being positive in the cases of  $\text{NaCl}$  and  $\text{KCl}$  and negative in the cases of  $\text{CaCl}_2$ ,  $\text{SrCl}_2$ , or  $\text{BaCl}_2$ . Lithium chloride increases the value of the coefficient of penetration, with a negative acceleration so that at concentrations lying above  $0.33 \text{ N}$  the algebraic sum of these opposite effects results in a decrease of the value of the coefficient of penetration.



## STUDIES OF AUTOLYSIS.

### IV. THE LATENT PERIOD IN AUTOLYSIS.

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In 1904 Lane-Claypon and Schryver<sup>1</sup> reported a latent period in autolysis amounting in the normal liver to about 4 hours. In the livers of fasting animals the latent period was absent or much shorter than in the well fed. If active trypsin preparations were introduced into the autolyzing liver mass, digestion began at once. These observations led the authors to the conclusion that in the living liver cell no free proteolytic enzymes existed; that the first stage of postmortem disintegration set free an active enzyme from a zymogen, and that the latent period represented, therefore, a survival period in the tissue. The authors further concluded that during fasting the zymogen was activated in some way, and thus autolysis intervened to allow the starving animal to utilize its own tissues for the development of energy by combustion, which otherwise would remain unavailable. The validity of this attractive hypothesis rests upon the rather limited observations reported in this one paper, and upon the interpretation of those observations. It has appeared to us worth while to examine further the early stages of autolysis of the liver in the hope that some new insight might be gained into the mechanism which starts and controls this important reaction. In the present paper we have made no attempt to test the differences between well fed and starving material, though both kinds are represented in our data. To a certain extent we have confirmed the findings of Lane-Claypon and Schryver, but our interpretation of the phenomenon is at variance with theirs. We have called attention to the great individual differences in appar-

<sup>1</sup> Lane-Claypon, J. E., and Schryver, S. B., *J. Physiol.*, 1904, xxxi, 169.



ently normal livers in a previous paper<sup>2</sup> and we have found that such differences are very apparent in the early stages of autolysis. Such differences we believe are undoubtedly associated with differences in the nutritional condition of the organ, and in a later paper we expect to present data upon this point.

*Experiment I.*—Three well fed rabbits were killed and bled. The livers were removed at once, ground to a fine pulp, divided equally, and each half was made up to 500 cc., with toluene water. 25 minutes after the death of the animal the initial samples were removed and the digests warmed quickly to 37°C. To one digest sufficient HCl was added to have made it a 0.02 N solution. Two sets of samples were taken, one precipitated by trichloroacetic acid as described in a previous paper,<sup>2</sup> the other coagulated by heat in the presence of  $\text{KH}_2\text{PO}_4$ . The samples from the acid digest were first treated with  $\text{CaCO}_3$  to neutralize the mineral acidity, an excess of  $\text{KH}_2\text{PO}_4$  was added, and the sample plunged into a boiling water bath. Boiling water was added till the volume approximated 90 cc. and the samples were kept at the boiling point 3 minutes after coagulation appeared to be complete. Control samples were coagulated in the same way. Heat-coagulated samples were cooled, made up to 100 cc., and filtered. The filtrate was analyzed for total nitrogen by the Kjeldahl method and for amino nitrogen by the Van Slyke micro method.

A survey of the figures shows: 1. The initial sampling is uncertain. The diffusion of water into the protein masses and the diffusion out of soluble nitrogenous compounds has not attained equilibrium. The initial samples are therefore subject to considerable error and their value must be interpreted in relation to the figures obtained in subsequent samples. We find the same irregularities in the initial values obtained by Schryver and we believe there is nothing significant in the low or high figures of the initial samples other than lack of equilibrium.

2. The non-coagulable nitrogen of the control shows a latent period of about 1 to 2 hours before unmistakable increase appears. In the acid digest, on the other hand, there is no latent period, or it is less than 30 minutes.

3. The Van Slyke method shows a latent period in the amino-acid appearance of 4 to 8 hours in the control, and of about 1 hour in the acid digest.

4. The trichloroacetic acid method shows increase of amino-

<sup>2</sup> Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, 1916, xxv, 261.

TABLE I.  
*Rabbit Livers.*

Time.	0	30 min	60 min	2 hrs.	4 hrs.	8 hrs.	27 hrs.	4 days.
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## Trichloroacetic acid method.

Cc. 0.2 N amino-acid per 6.25 cc. tissue suspension

I. Control.....	0.25	0.30	0.30	0.30	0.50	0.60	0.70	0.80
II. " HCl.....	0.30	0.30	0.40	0.40	0.70	1.30	2.40	3.20

## Non-coagulable N.

Cc. 0.2 N  $\text{NH}_3$  per 6.25 cc. tissue suspension.

I. ....	1.75	1.60	1.90	2.00	2.15	2.20	2.30	2.45
II. ....	1.80	2.20	2.20	3.20	3.30	4.90	6.30	8.60

## Van Slyke method.

Mg. N per 1 cc. filtrate or 0.25 cc. tissue suspension.

I. ....	0.076	0.060	0.060	0.060	0.076	0.082	0.087	0.114
II. ....	0.054	0.049	0.087	0.103	0.157	0.211	0.477	0.604

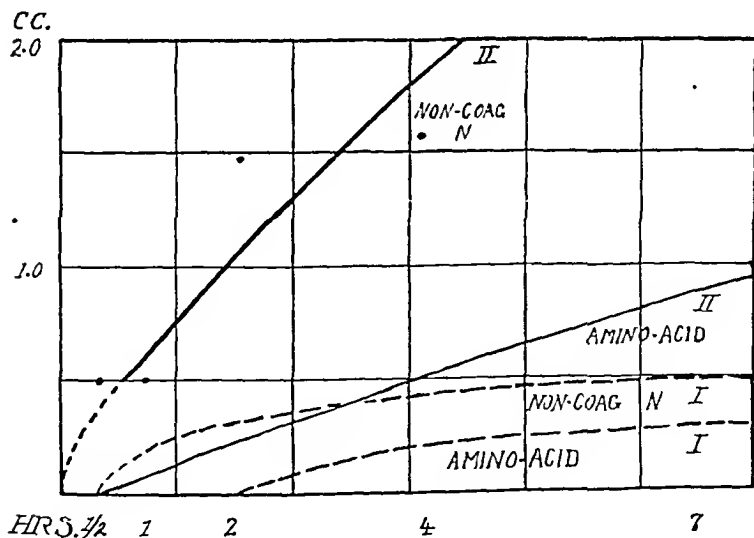


FIG. 1.

acids in the control after 2 to 4 hours, in the acid digest after 1 to 2 hours.

The figures and curves clearly indicate a lag between the appearance of increased non-coagulable nitrogen and the appearance of amino-acids. The lag would, therefore, appear to express the time required in progressive hydrolysis of native proteins to reach, first, a measurable increase of non-coagulable protein, and second, a measurable increase of amino-acids. If amino-acids are split off during the earlier stages of the reaction they are not measurable by the technique employed in this experiment. It should be noted that Schryver used heat coagulation with trichloroacetic acid present to throw down his undigested material; his figures therefore are comparable with those obtained by us with trichloroacetic acid precipitation. In this particular case our results agree very well with Schryver's. It is quite possible therefore that Schryver's latent period was nothing more significant than the time required between the onset of autolysis and the appearance of the final cleavage products.

In all cases the presence of acid cuts down the latent period, just as it has been shown to increase the rate and extent of hydrolysis in longer digestions. The acid digest reaches approximate equilibrium in 4 days, at four times the amino-acid level of the control (3.2 to 0.8). This difference in equilibrium represents the difference in the actual substratum available for the enzymes in the two digests. Such a difference in mass of substratum should cause proportionate differences in the rate of change at the start, and we find in fact that the time required to show amino-acid increase is about four times as long in the control as in the acid digest (4 hours to 1 hour). If we assume, with Schryver, that a certain time elapses before the proteases present in the control are activated (about 4 hours), whereas in the acid digest the enzyme is at once active, then a much greater difference should exist between the time of amino-acid increase in the acid and control digests. The differences between the control and the acid digest do not express differences in the time of enzyme activation, but are expressive of the same differences which obtain throughout the continued hydrolysis, determining rate of change and point of equilibrium. These differences appear to us to be indicative of different masses of substratum only.

In one of Schryver's experiments trypsin was added to a fresh liver digest. Hydrolysis was apparent at once, while in the control there was the usual 4 hour latent period. But to trypsin practically all the liver proteins are substratum, while to the liver proteases only a small fraction—about 25 per cent on the average—is available for the reaction. With this difference in the specificity of the two enzymes, and with no means of knowing the amount of trypsin added compared to the proteases already there, a comparison of these two digests is without significance and certainly does not prove Schryver's contention that the latent period represents the time elapsed before the enzyme becomes active.

*Experiment II. The Amino-Acid Lag in Rapid Autolysis.*—Liver obtained from an old horse and removed within a minute of the death of the animal, was sliced, chilled to  $-10^{\circ}\text{C}.$ , and kept frozen till ground to a snow and mixed with toluene water at  $0^{\circ}\text{C}.$  Samples were taken during a 10 minute interval, the time required to bring the digest to  $37^{\circ}\text{C}.$ , acid was added, and sampling continued at short intervals. Van Slyke and Kjeldahl figures were obtained on the heat coagulation filtrates.

TABLE II.  
*Horse Liver.*

Time.	Van Slyke method Mg N per 1 cc filtrate or 0.25 cc tissue suspension	Non-coagulable nitrogen. Cc 0.2 N $\text{NH}_3$ per 6.25 cc tissue suspension
<i>min.</i>		
0	0.095	1.70
10	0.111	1.70
12	0.106	2.05
15	0.123	2.30
20	0.128	2.60
25	0.128	3.00
30	0.128	3.10
35	0.145	3.20
40	0.145	3.30
50	0.159	3.60
60	0.162	3.70
70	0.173	3.85

In this case, we find immediate response to the addition of acid in the total nitrogen, with a delayed response in the amino-acid figures. Both curves rise most rapidly at the start, and are of

the logarithmic type. If acid is the activator of the enzymes, as Morse has recently maintained,<sup>3</sup> then at the moment of the addition of HCl the enzymes were completely active. Still we find the lag between early and late products of autolysis. In other words, ruling out any possible difference in the time of activation, we still find a typical latent period when we measure amino-acids. *In this case, then, it is certain that the latency of the reaction does not indicate activation, but merely reaction lag, which is sufficient to account for Schryver's results.*

*Experiment III. The Effect of Added Substratum.*—The same material was used as in Experiment II. Gelatin solution was added as an available foreign protein. It was boiled with  $\text{CaCO}_3$  for several hours and filtered, thus insuring neutrality. The gelatin was found to digest at once with no clear indication of a latent period. Making due allowance for the errors of end-point in the titrations, there was undoubted increase in amino-acids within 60 minutes; in the control equally certain increases were not found until 4 to 8 hours had elapsed.

In this case, then, we have abolished the latent period, or cut it down largely, by a procedure which was designed to eliminate a possible activating action of acids. By simply increasing the mass of substratum, itself neutral, we have duplicated the effect on the early stages of autolysis produced by adding mineral acids. If the possibility of an acid activation latency can be abolished by increasing the mass of substratum, then the enzyme must either have been active already, or the gelatin activated it—an improbable alternative. It seems clear in this case that an acidity not above that of the normal living tissue is all that is necessary to demonstrate activity of the enzyme provided there is any appreciable amount of substratum present.

*Experiment IV. The Effect of Reaction.*—The material was the same as that used in Experiment II. Two sets of samples were taken as before. The Van Slyke figures are upon 1 cc. of filtrate and represent 0.25 cc. of the digest. The non-coagulable nitrogen figures are upon 25 cc. of the same filtrates and represent 6.25 cc. of the digest.

The early readings with the Van Slyke apparatus were all small and subject to rather large errors in our hands. They indicate but slight increase of the amino nitrogen until the 24 hour period.

<sup>3</sup> Morse, M., *J. Biol. Chem.*, 1916, xxiv, 163.

TABLE III.

*Trichloroacetic Acid Method.**Cc. 0.2 x Amino-acid per 6.25 Cc. Tissue Suspension.*

Time	0	15 min	30 min	60 min	90 min	2 hrs	4 hrs	8 hrs	24 hrs
I. Control..	0 10	0 50	0 50	0 50		0 55	0 50	0 60	0 65
II. Gelatin..	0 80	0 85	0 90	0 95	0 95	1 05	1 15	1 35	1 70

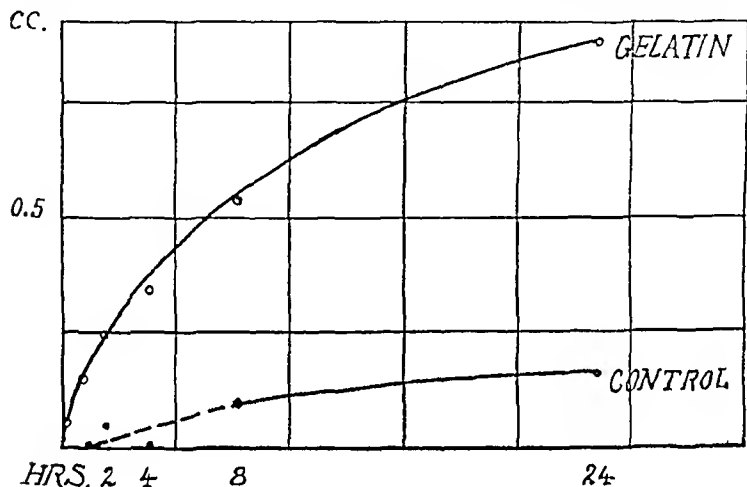


FIG. 2

In the acid mixtures the increase occurs earlier. In the alkaline mixtures the figures are irregular, but digestion is slight until the 24 hour period, and is generally less as the bicarbonate is increased. The neutral phosphate also inhibits the digestion markedly.

In the trichloroacetic acid filtrates a clearly defined latent period is difficult to recognize. Some digestion appears to be under way by the end of an hour—assuming that the correct figure for the initial samples is about 0.45 cc. With acid present the lag is decreased in proportion to the amount added. In the alkaline samples there appears to be digestion about like that of the control, or somewhat less. The phosphate mixtures behave in the same way.

TABLE IV

Content of digestion bottles		0	15 min	30 min	60 min	90 min	2 hrs	4 hrs	8 hrs	24 hrs	3 days
Van Slyke method.											
Mg N per 1 cc. filtrate or 0.25 cc tissue suspension											
I	Control	0.07		0.07	0.07		0.08		0.07	0.08	0.11
II.	" HCl to 0.01 N.	0.07	0.07	0.07	0.09	0.11	0.11	0.13	0.19	0.28	0.35
III	" " " 0.02 "	0.07		0.07	0.11	0.15		0.23	0.24	0.43	0.60
IV	" " " 0.04 "	0.07	0.07	0.06	0.12	0.19	0.19	0.29	0.40	0.56	0.75
V	" NaHCO <sub>3</sub> 0.5 gm	0.07			0.08				0.07	0.08	0.09
VI	" " 1.0 "								0.08	0.07	0.09
VII	" " 2.0 "	0.06					0.06	0.07	0.08	0.07	0.07
VIII	" K <sub>2</sub> HPO <sub>4</sub> 5.0 "	0.08						0.07	0.09		0.07
IX	" " 2.0 "	0.06							0.07	0.06	

## Non-coagulable nitrogen

Cc 0.2 N NH<sub>3</sub> per 6.25 cc tissue suspension

I	Control	2.4		1.6	1.6	1.6	1.7	1.9	1.8	2.1	2.7
II	" HCl to 0.01 N	2.2	1.7	1.9	2.0	2.2	2.2	2.7	3.3	3.0	5.4
III	" " " 0.02 "	2.2		2.3	2.6	2.9	3.3	4.0	5.4	6.7	8.6
IV	" " " 0.04 "	2.2	2.7	3.0	3.7	4.5	4.9	6.0	7.6	8.7	10.7
V	" NaHCO <sub>3</sub> 0.5 gm	1.5					1.6	1.7	1.7	1.8	2.2
VI	" " 1.0 "	1.9					1.7		1.7	1.8	2.2
VII	" " 2.0 "	1.6						1.9	2.0	1.9	2.3
VIII	" K <sub>2</sub> HPO <sub>4</sub> 5.0 "	1.8					1.6	1.7	1.7	1.8	2.3
IX	" " 2.0 "	1.7					1.7	1.7	1.7	1.8	2.5

## Trichloroacetic acid method

Cc 0.2 N amino-acid per 6.25 cc tissue suspension

I	Control	0.25		0.45	0.50	0.50	0.55		0.60	0.80	1.00
II	" HCl to 0.01 N.	0.35	0.40	0.45	0.55	0.60	0.65	0.90	1.25	1.75	
III	" " " 0.02 "	0.40	0.40	0.45	0.65	0.80	0.80	1.10	1.60	2.45	
IV	" " " 0.04 "	0.50	0.45	0.55	0.75	0.95	1.10	1.50	2.15	2.80	4.0
V	" NaHCO <sub>3</sub> 0.5 gm	0.40		0.45	0.45		0.60			0.70	0.85
VI	" " 1.0 "	0.35		0.45	0.40		0.65		0.60	0.80	0.90
VII	" " 2.0 "	0.35		0.40	0.40			0.55		0.60	0.80
VIII	" K <sub>2</sub> HPO <sub>4</sub> 5.0 "	0.40		0.45	0.45			0.50	0.50	0.70	0.80
IX	" " 2.0 "	0.40		0.45	0.40	0.50	0.60	0.60	0.60	0.70	0.80

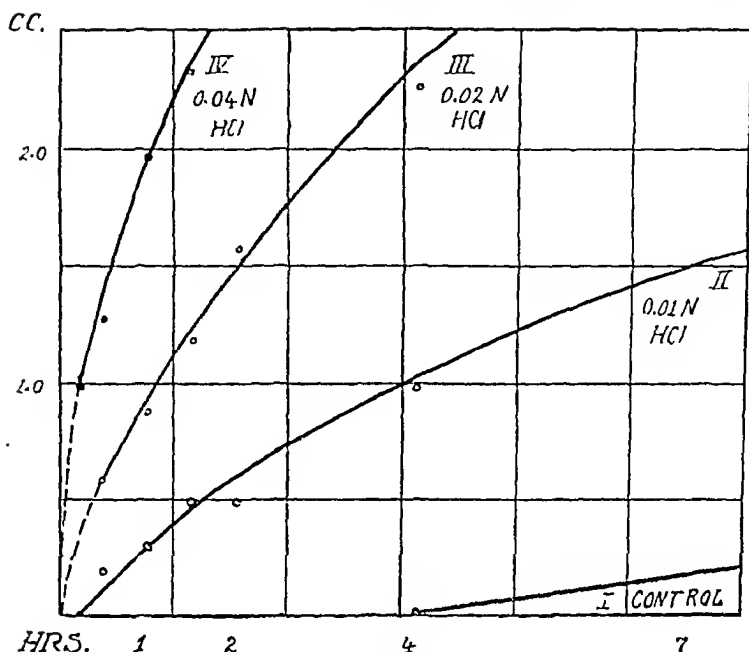


FIG. 3.

Non-coagulable nitrogen shows a lag in the control of about 2 to 4 hours before digestion is definitely seen. Increasing acidity cuts down this lag so that the most acid sample is clearly moving within 15 minutes. The figures on the alkaline or salt samples are irregular but appear to indicate a lag of 4 to 8 hours before digestion is definitely under way.

Despite the rather large irregularities of our figures in this series, which we believe represent inevitable irregularities of sampling, of equilibrium by diffusion, and of heat coagulation, the latent period appears to be shortened by acids, and lengthened by neutrality or diminished acidity. None of the digests were permanently alkaline to litmus.

*Experiment V.*—Liver obtained from a healthy well nourished calf was frozen and ground to a snow. All digests were sampled and then rapidly warmed to 37°C.



TABLE V

Content of digestion bottles	0	15 min	30 min	60 min	90 min	2 hrs	4 hrs	8 hrs	24 hrs
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## Van Slyke method

Mg N per 1 cc filtrate or 0.25 cc. tissue suspension.

I Control	0.04		0.04	0.06	0.07	0.07	0.07	0.08	0.09
II " HCl to 0.04 N	0.04	0.06	0.08	0.09	0.10	0.12	0.14	0.17	0.25
III " 0.25 gm. NaHCO <sub>3</sub>	0.04		0.06	0.06	0.06	0.07	0.07		0.12
IV. " 0.50 " "	0.04		0.06	0.06	0.06	0.06	0.07	0.08	0.08
V " 1.00 " "	0.04		0.04	0.06	0.05	0.06		0.06	0.07
VI " gelatin	0.10		0.12	0.13	0.15	0.16	0.18	0.20	0.25

## Non-coagulable nitrogen.

Cc 0.2 N NH<sub>3</sub> per 6.25 cc tissue suspension

I Control	1.7		1.8	1.8	2.05	2.10	2.15		2.30
II " HCl to 0.04 N	1.7	2.4	2.8	2.95	3.00	3.60	4.00	4.30	5.30
III " 0.25 gm NaHCO <sub>3</sub>	1.7		1.70	1.90		1.90	1.95	2.00	2.20
IV. " 0.50 " "	1.8		1.70	1.75	1.80	1.75	1.95	1.90	2.10
V " 1.00 " "	1.70		1.60	1.65		1.70	1.80	1.80	2.00

## Trichloroacetic acid method.

Cc 0.2 N amino-acid per 6.25 cc tissue suspension

I Control	0.45		0.45	0.45	0.55	0.60	0.65	0.70	0.85
II " HCl to 0.04 N	0.40	0.45	0.50	0.55	0.70	0.70	0.80	1.05	1.50
III " 0.25 gm NaHCO <sub>3</sub>	0.50			0.55	0.50	0.55	0.60	0.70	0.75
IV " 0.50 " "	0.45			0.50		0.55	0.60	0.65	0.70
V " 1.00 " "	0.45			0.50		0.50	0.55	0.60	0.65
VI " gelatin	1.10		1.20	1.25	1.35	1.40	1.50	1.70	2.30

1. The non-coagulable nitrogen of the control appears rapidly, increasing within 90 minutes or less. The amino-acid nitrogen parallels the non-coagulable nitrogen in this case.

2. Acidity cuts down the latent periods. A lag is shown between the non-coagulable and amino-acid nitrogen by the trichloroacetic acid method.

3. In the presence of NaHCO<sub>3</sub> the non-coagulable nitrogen increase is delayed from 2 to 8 hours. On the other hand, the Van Slyke figures are irregular and show only a doubtful delay over the control until the 24 hour sample. The trichloroacetic acid figures show a more distinct delay in amino-acid production and a decided inhibition of the digestion all the way through

4. The addition of neutral gelatin gives a reaction curve almost identical with the curve of the acid digest (in both trichloroacetic acid and Van Slyke methods). The experiment is in the nature of a coincidence, where the amount of substratum produced by the addition of acid was identical with the increase due to neutral gelatin. It gives strong confirmation to the belief that the question of activation of an enzyme is subsidiary in the mechanism which starts and controls autolysis and that the determinative factor is the amount of substratum present and the conditions which modify that amount in the living cell.

It was of interest to note that the glycogen turbidity of the control filtrates persisted even after 24 hours, though with constant diminution. In the acid samples and in the gelatin digest the glycogen of the filtrates disappeared much more rapidly.

*Experiment VI.*—Liver obtained from a 5 year old horse was removed within 2 minutes of the death of the animal and frozen at once.

TABLE VI.

*Trichloroacetic Acid Method.**Cc. 0.2 N Amino-Acid per 6.25 Cc. Tissue Suspension.*

	0	15 min	30 min	45 min	60 min	90 min	2 hrs	4 hrs	8 hrs	26 hrs.	3 days.
I. Control...	0 30		0 35		0 45	0 45	0 55	0 60	0 60	0 65	1 00
II. " gelatin	0 65	0 60	0 60	0 60	0 70	0 80	0 85	0 95	1 05	1 30	2 25
III. " 0.5 gm. NaHCO <sub>3</sub> .	0 30		0 35		0 35	0 35	0 40	0 40	0 50	0 55	0 65
IV. Control 0.5 gm. NaHCO <sub>3</sub> gelatin	0 70	0 60	0 70	0 60	0 60	0 70	0 70	0 90	0 95	1 10	1 50
V. Control HCl to 0.04 N	0 30	0 65	0 70	0 80	0 70	1 00	1 15	1 45	1 80	2 60	4 00
VI. " 1.0 gm. NaHCO <sub>3</sub>	0 30		0 40		0 35	0 35	0 45	0 45	0 45	0 55	0 60

1. The control shows a latent period of about 60 to 90 minutes.

2. Gelatin shows digestion within 60 minutes.

3. Acid causes digestion within 15 minutes, far more rapid and extensive in this case than where gelatin was present. Though the same amounts of gelatin and acid were used as in Experiment V, the conditions are far from coincident in this liver.

4. Bicarbonate increases the latent period and the autolysis is very slight even in 3 days.

5. Gelatin added to a bicarbonate mixture reduces its latent period.

An analysis of the figures beginning with the 8 hour samples shows that gelatin is digesting in both normal and salt mixtures. At the end of 8 hours the digestion rate is indistinguishable, but in 26 hours the digestion of gelatin is evidently slower in the salt mixture. The 3 day sample also shows a certain amount of inhibition of the gelatin digestion. These figures bear out the findings reported in a previous paper<sup>2</sup> where the digestion of gelatin was found to be inhibited by alkalis in long continued autolysis, but where the inhibition was less than upon the digestion of liver proteins.

TABLE VII.  
*Analysis of Figures of Table VI.*

Time	8 hrs.		26 hrs.		3 days.	
	Gain.	Digestion of gelatin.	Gain.	Digestion of gelatin.	Gain.	Digestion of gelatin.
I ..	0 25		0.30		0.65	
II..	0 45	0.20	0.70	0.40	1.60	0.95
III.....	0 15		0.20		0.30	
IV.....	0 35	0 20	0.50	0.30	0.90	0.60

These preliminary studies of the early stages of autolysis warrant the following conclusions:

1. The time required for the appearance of measurable amounts of amino-acids is greater than that required for the increase in non-coagulable nitrogen. The latent period reported by Lane-Claypon and Schryver is to be explained, therefore, as the lag between the initial stages of proteolysis and the final products.

2. The addition of the optimum amount of acid may reduce the lag to a very few minutes, from the usual 4 hours. At the same time the increase of non-coagulable nitrogen may be too rapid to show any latent period.

3. The addition of neutral gelatin in proper amount to a fresh autolyzing liver may reduce the latent period identically with acid. In this case the enzyme is proven to be active in the neutral or amphoteric liver material before any appreciable amount of acid has been formed in the mixture.

4. Since gelatin can hardly be an activating agent, and since its effect on the digest as a whole is identical with that of increased acidity, confirmation is found for the assumption that the effect of acid on liver material is to increase the mass of substratum in the mixture.

5. There is nothing in the data presented here which suggests the activation of an enzyme as an important determinative step in autolysis. If the enzyme is activated it takes place at a  $H^+$  level such as is found in the living tissue. Furthermore, none of the curves show any indication of an autocatalytic phase in the early stages of the reaction, such as Morse assumed,<sup>3</sup> due to activation of the enzyme by developing acidity.

Length of the latent period, rate of proteolysis as measured by concentration of products, and final equilibrium of the digestion all appear to depend on the mass of substratum available for hydrolysis by the proteases present normally in the liver cell.



# CHEMICAL STIMULATION OF NERVES.\*

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## I.

In 1899 Loeb<sup>1</sup> stated that electrolytes cause twitchings of muscles, while non-electrolytes have no such effect. But not all electrolytes were equally efficient as stimulants. The sodium salts seemed the best adapted for this purpose and their efficiency varied considerably with the nature of the anion, as the following list shows.<sup>2</sup>

TABLE I.

Salt.	Minimal stimulating concentration.	Salt.	Minimal stimulating concentration.
NaCl.....	M/16	Na <sub>2</sub> succinate.....	M/16
NaBr.....	M/16-M/32	Na <sub>2</sub> SO <sub>4</sub> .....	M/32
NaI.....	M/32	NaHCO <sub>3</sub> .....	M/16-M/32
Na acetate.....	M/32-M/64	Na <sub>2</sub> oxalate.....	M/260
NaF.....	M/64-M/96	Na <sub>2</sub> HPO <sub>4</sub> .....	M/128
Na formate.....	M/80-M/160	Na <sub>3</sub> citrate.....	M/200

Since Ca inhibits the twitchings caused by NaCl, as Ringer first observed, Loeb suggested that the higher stimulating effect of oxalates, fluorides, and phosphates might be due to a precipitation of Ca in the tissues, but that this could not account for the high stimulating effect of certain other salts like Na formate.

In his studies on the chemical stimulation of nerves Mathews<sup>3</sup>

\* These experiments were commenced in 1912 and completed during the year 1915-16. On account of the war it was impossible to submit the paper with the more recent results to Dr. Ewald before publication.

<sup>1</sup> Loeb, J., Festschrift f. Prof. Fick, 1899, p. 101.

<sup>2</sup> Loeb, *Arch. ges. Physiol.*, 1902, xci, 243.

<sup>3</sup> Mathews, A. P., *Am. J. Physiol.*, 1904, xi, 455.

found similar stimulating values for the sodium salts upon the motor nerve (sciatic of the frog).

TABLE II.

Salt.	Minimal stimulating concentration.	Salt.	Minimal stimulating concentration.
NaCl.....	M/12	Na <sub>2</sub> SO <sub>4</sub> .....	M/32
NaBr.....	M/12	NaHCO <sub>3</sub> .....	M/5
NaI.....	M/12	Na <sub>2</sub> oxalate.....	M/32
Na acetate.....	M/12	Na <sub>2</sub> HPO <sub>4</sub> .....	M/35
NaOH.....	M/20	Na <sub>3</sub> citrate.....	M/50

The highest stimulating efficiency was that found for Na<sub>3</sub> citrate. On the basis of these and other experiments Mathews put forward the hypothesis that only the anions have a stimulating effect, while the cations have a depressing effect. The stimulating effect, he assumes, runs parallel to the solution tension of the anion. This theory meets with serious difficulties, since, first, it leaves unexplained why Ba salts have a higher stimulating effect than Na salts, and, second, why, as Loeb found, the stimulating effect of NaCl upon muscle is increased when some acid is added. Moreover, it is strange that while in NaCl the stimulation of the nerve may have a latent period of several hours, in the case of citrates the latent period may be only a few minutes. This time element indicates that another factor must enter into these results, and we shall see that this is probably the rapidity of diffusion of the salt into nerve or muscle. In 1899 Loeb had explained the accelerating action of acid upon the stimulating effect of NaCl upon muscle in this way. We have made a series of experiments which are not compatible with Mathews' theory, but which support the diffusion theory. According to this theory, the stimulating action of sodium salts upon nerve and muscle is inhibited by Ca and Mg, not on account of the depressing effect of the latter, as Mathews assumes, but because the Ca and Mg prevent the diffusion of salt into these organs.

## II.

NH<sub>4</sub>Cl is not able to stimulate nerve or muscle. The citrate of this base may have some stimulating action and this fact would

agree with Mathews' theory that the stimulating action depends only on the anion. We have found, however, that if in the molecule  $\text{NH}_4\text{Cl}$  the four hydrogen atoms are replaced by the ethyl group the resulting salt [tetraethylammonium chloride,  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$ ] has a much higher stimulating effect upon the motor nerve than any sodium salt, even sodium citrate. As to our method, it may suffice to state that only the nerve was immersed in the solution, and the muscle was carefully protected from contact with the solution. The nerve muscle preparations (sciatic nerve and gastrocnemius muscle) from *Rana pipiens* were used. The solution was always made isotonic with an  $\text{m}/8$   $\text{NaCl}$  solution by the addition of the proper amount of cane sugar. The experiments were carried out in moist chambers and the exposed part of the nerve was carefully protected against drying out through frequent wetting with Ringer solution. The muscles were weighted. We used Merck's tetraethylammonium chloride,<sup>4</sup> while the other salts were Kahlbaum's.

Table III gives a comparison between the stimulating efficiency of corresponding salts of  $\text{N}(\text{C}_2\text{H}_5)_4$  and of  $\text{Na}$ . Our values for the sodium salts do not entirely agree with those given by Mathews. We found that a  $\text{m}/12$   $\text{NaCl}$  solution is too weak to stimulate the nerve. In order to produce muscular contractions with certainty we had to immerse the nerve in  $\text{m}/4$   $\text{NaCl}$ . An  $\text{m}/8$   $\text{NaCl}$  solution was only in exceptional cases able to induce twitchings through the nerve. On the other hand, we found  $\text{Na}_3$  citrate more efficient than in Mathews' experiments.

TABLE III

Minimal stimulating concentrations of salts of  $\text{Na}$  and  $\text{N}(\text{C}_2\text{H}_5)_4$  \*

$\text{NaCl}$ ..	$>\text{m}/8$	$\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$	about $\text{m}/160$
$\text{NaOH}$ .	$\text{m}/20$	$\text{N}(\text{C}_2\text{H}_5)_4\text{OH}$	" $\text{m}/200$
$\text{Na}_3$ citrate	$\text{m}/100$	$(\text{N}(\text{C}_2\text{H}_5)_4)_3$ citrate	" $\text{m}/160$

\* In the hypotonic solution of these substances the solution was made isotonic with  $\text{m}/8$   $\text{NaCl}$  through the addition of cane sugar.

These data show clearly, first, that the stimulating effect of the tetraethylammonium salts is equal to or greater than that of

<sup>4</sup> In reality Merck's tetraethylammonium hydroxide was transformed into the chloride by adding  $\text{HCl}$  to the point of neutrality.



sodium citrate; and second, that the nature of the anion has little influence upon the efficiency of the tetraethylammonium salts. The powerful stimulating action of tetraethylammonium chloride is therefore due to the cation and not to the anion.

That it is the cation which determines the stimulating efficiency in this case is further proved by the fact that if the methyl group is substituted for the ethyl group the efficiency is lost. Tetramethylammonium chloride was ineffective in solutions isotonic with the nerve, as were also ammonium chloride, methylamine hydrochloride, ethylamine hydrochloride, diethylamine hydrochloride, and piperidine hydrochloride.

Attention should also be called to the fact that only strong bases act as nerve stimulants, while weak bases, such as  $\text{NH}_4\text{OH}$  and the amines, *e.g.*, diethylamine, triethylamine, butylamine, and benzylamine, are ineffective (except in hypertonic concentrations). This is almost the reverse of the relative effects of weak and strong bases in inducing artificial parthenogenesis.<sup>5</sup> It may be that the amines injure the nerve more than they do the egg.

### III.

If the inhibitory effect of calcium salts upon the stimulating effect of sodium salts were due to an antagonism between anion and cation, as Mathews assumes, the amount of Ca required to suppress the stimulating action of a salt should depend chiefly upon the nature of the anion of the stimulating salt. If the stimulating action of tetraethylammonium chloride is due to the chloride, the amount of Ca required to suppress the stimulating action of this compound should be of the order of that required for the suppression of the stimulating action of NaCl. The contrary was found to be the case.

Solutions of sodium salts and of tetraethylammonium chloride were prepared so that with the proper addition of cane sugar the solution was always isotonic for the nerve. To 100 cc. of such a solution were added 1 or more cc. of  $\text{M}/8$   $\text{CaCl}_2$  solution. The nerve was put into the solution and the number of cc. of  $\text{M}/8$   $\text{CaCl}_2$  were observed that had to be added to 100 cc. of the stimulating solutions in order just to inhibit all the twitchings of the muscle when the nerve alone was immersed.

<sup>5</sup> Loeb, *Artificial Parthenogenesis and Fertilization*, Chicago, 1913.

TABLE IV.

Stimulating solution.	Cc. of M/8 CaCl <sub>2</sub> added to 100 cc. of stimulating solution.	
	Complete inhibition of twitchings.	No or incomplete inhibition of twitchings.
M/4 NaCl.....	2	1
M/8 Na <sub>2</sub> SO <sub>4</sub> .....	3.5	2, 2, 1, 1.5, 2.5, 3
M/64 Na <sub>2</sub> citrate.....	3, 5, 3, 5, 4	2, 3, 3.5
M/40 " " .....	7.5, 7.5	5, 6
M/32 " " .....	12.5, 12.5, 12.5, 12.5, 12.5	7.5, 10, 11, 10, 7.5
M/24 " " .....	20, 20, 22.5	7.5, 10, 12, 15, 17.5, 19, 20
M/16 " " .....	30	25, 20
M/64 Tetraethylammonium chloride.....	12.5	7.5, 10
M/40 Tetraethylammonium chloride.....	12.5	5, 7.5, 10
M/32 Tetraethylammonium chloride.....	12.5	7.5, 10, 12.5
M/24 Tetraethylammonium chloride.....	20	17.5, 15, 12, 10

There can be no doubt that the amount of CaCl<sub>2</sub> required for the suppression of twitchings in tetraethylammonium chloride is of a much higher order of magnitude than that required for the suppression of twitchings in sodium chloride. While the addition of 2 cc. of M/8 CaCl<sub>2</sub> suffices for this suppression in 100 cc. of M/4 NaCl, 12.5 cc. of M/8 CaCl<sub>2</sub> are required for the suppression of twitchings in a M/40 tetraethylammonium chloride solution, which is sixty times as much in the latter as in the former solution. This result is incompatible with the idea that the antagonism exists in both cases between the Cl<sup>-</sup> and the Ca<sup>++</sup> ion.

The amount of Ca required for the tetraethylammonium chloride solution is nearer the order of magnitude of that required for the sodium citrate solution.

Not only Ca but Mg also suppresses the twitchings of the nerve. The concentration of Mg required for complete inhibition of the stimulating effect of citrates and tetraethylammonium chloride is much greater than that of Ca, as Table V shows.

## V.

We now come to the discussion of a crucial question for the problem of chemical stimulation; namely, does the Ca inhibit the stimulating effect of  $N(C_2H_5)_4Cl$  or of  $Na_3$  citrate, on account of a "depressing" action, *i.e.*, by lowering the irritability of the nerve or merely by preventing these salts from diffusing sufficiently rapidly through the sheath of Schwann to the medullary sheaths or the axis cylinders inside the sheath? It has been proved sufficiently by experiments on *Fundulus* that, in this latter case at least, the antagonistic effect of  $CaCl_2$  upon  $NaCl$  is due to the  $CaCl_2$  preventing the diffusion of sodium salts through the membrane.<sup>7</sup>

It can first be shown that the Ca does not inhibit the efficiency of the stimulating salt by depressing the irritability of the nerve. The original threshold of two nerves of the same frog was 45 and 51. The one was put into  $m/24$   $Na_3$  citrate in sugar, the other in 100 cc.  $m/24$   $Na_3$  citrate + 20 cc.  $m/8$   $CaCl_2$  in sugar (sufficient to make the solution isotonic). In the latter solution no twittings occurred (in  $3\frac{1}{2}$  hours). The threshold rose to 57 in 40 minutes and was still 49 after  $3\frac{1}{2}$  hours. In the solution without Ca violent contractions began in 5 minutes when the threshold had risen to 58, and lasted 40 minutes, when the threshold was 54. In an hour the nerve was no longer irritable. It is natural to reason that the Ca prevented the citrate from diffusing into the nerve and the latter remained normal; while the nerve in the solution free from Ca was stimulated because the citrate diffused into the nerve which was killed on account of such diffusion. When the nerve, that had not twitched for 3 hours in the citrate solution containing Ca, was put into the citrate solution without Ca, twittings began in 33 minutes and lasted for 20 minutes. During the time of twitching the irritability had risen from 49 to 60, due probably to the diffusion of the  $Na_3$  citrate into the nerve. This was confirmed in a large number of experiments. No precipitate occurred in the concentration in which citrate and Ca were used.

The same was observed in the experiments with tetraethylam-

<sup>7</sup> Loeb, *Arch. ges. Physiol.*, 1905, cvii, 252; *Science*, 1912, xxxvi, 637; *Biochem. Z.*, 1912, xlvii, 127.

monium chloride. The addition of enough Ca to prevent the twitchings did not lower the threshold of faradic irritability. This eliminates the idea of Mathews that the inhibiting effect of Ca is due to a depression of the irritability of the nerve.

There is direct evidence, however, that the addition of Ca retards the rapidity of diffusion of the stimulating salts into the nerve. *If we add a considerable quantity of  $\text{CaCl}_2$  to a stimulating salt solution, but not quite enough to inhibit the stimulating effect, we find that the latent period of stimulation is considerably lengthened.* The results on two nerves of the same frog were always compared (Table VI).

TABLE VI.

*Latent Period for Stimulation of the Nerve in Tetraethylammonium Chloride.*

Without calcium		With calcium	
	min	cc	min
M/64 $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$	6	+ 5 0 M/8 Ca	16
	2	+ 7 5 "	70
	5	+10 0 "	35
M/32 " . . .	1	+ 7 5 "	11
	2	+10 0 "	35
	4	+12 5 "	48
M/24 "	3	+17 5 "	78
	5	+15 0 "	21

The addition of Ca to  $\text{Na}_3$  citrate acts in a similar way. When enough Ca is added the latent period of stimulation of the nerve by  $\text{Na}_3$  citrate is increased. Thus in M/24  $\text{Na}_3$  citrate the latent period was in five experiments between 5 and 8 minutes; in 100 cc. M/24  $\text{Na}_3$  citrate + 12.5, 15.0, 17.4, and 19 cc. M/8  $\text{CaCl}_2$ , the latent periods were 23, 33, 40, and 34 minutes. Needless to say no precipitation of Ca citrate occurred in these mixtures.

It is, therefore, certain that Ca does not inhibit by a depression of the irritability of the nerve, but may inhibit by a diminution of the rate of diffusion of the stimulating salts into the nerve.

In addition it was found that when tetraethylammonium chloride solutions of different concentrations were tried, the latent period of stimulation for the nerve diminished with increasing concentration. Thus in M/32  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  the latent period averaged 2

minutes, while in M/64, 6, 2, 5, 6, 10, 3, and 14 minutes were observed. When too little Ca was added the increase in the latent period was often not noticeable.

## VI.

If we consider the idea that the Ca inhibits the chemical stimulation of the nerve by inhibiting the diffusion of the stimulating salts through the sheath of Schwann (or other envelopes) we must ask the question whether we cannot find an explanation for the great stimulating effect of certain salts like the citrates and those of  $N(C_2H_5)_4$  on a similar basis. It can be pointed out that the stimulating action of the salts as shown in Tables I and II indicates that those salts which precipitate Ca contained at the surface of cells have also a greater stimulating power. This would concern the oxalates and fluorides, but not the citrates, or  $N(C_2H_5)_4Cl$ . The question may arise whether after all these latter salts which are powerful nerve or muscle stimulants without precipitating Ca are not those which possess a comparatively high degree of solubility in organic substances such as lipoids of the nerve or the envelopes of the muscles.

If it could be shown that the degree of solubility and permeability decides the stimulating value of these salts, we can understand why both cations as well as anions stimulate the nerve, and why the cation  $N(C_2H_5)_4$  and the citrate anion should both have a high stimulating value. The diffusion of citrate into nerve and muscle is probably enhanced by the alkaline reaction of this solution.

## VII.

This would still leave it unexplained why oxalates, citrates, and  $N(C_2H_5)_4$  cannot stimulate the nerve after its irritability has been diminished by a longer treatment with a sugar solution; and why the addition of NaCl is required to make these salts efficient. As we have stated above, the NaCl is needed to bring the irritability of the nerve to a high value again. This proves that the nerve stimulants are only able to stimulate a highly irritable nerve; and that even a slight lowering of the electric irritability of the nerve renders these salts ineffective; although the nerve may still respond vigorously to galvanic currents of sufficient intensity.

The writer wonders whether the phenomena of chemical stimulation may not after all be cases of electrical stimulation which find their explanation in the electromotive phenomena described by Loeb and Beutner.<sup>8</sup> These authors observed that if we lead off from two spots of the surface of an intact animal or plant organ with two solutions of the same nature but of different concentration we notice the existence of considerable potential differences and these differences of potential follow Nernst's logarithmic formula. Such differences of potential originate also when we lead off from two substances of equal concentration but of different fat solubility. These E.M.F. originate at the boundary of watery and water-immiscible phases, and Beutner has developed their theory.<sup>9</sup>

When we immerse a nerve in a solution of  $\text{Na}_3$  citrate or  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  these substances will probably not diffuse with equal rate through every spot of the sheath of Schwann. It will thus happen that at one spot at the surface of the medullary sheath of an axis cylinder a certain amount of  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  solution will collect, while a neighboring spot of the same axis cylinder contains this solution in a much lower concentration or not at all. This will necessitate a potential difference which will give rise to a minute current. If the irritability of the axis cylinder is high enough this will give rise to a twitching in the corresponding muscle fibers. It agrees with this view that the twitchings of the muscle due to chemical stimulation of its nerve are extremely irregular, which would correspond to the possible irregularities in the diffusion of these substances between the nerve fibrils. On this assumption we can also understand why only electrolytes can cause a chemical stimulation. The currents observed by Loeb and Beutner have nothing to do with the so called current of injury since they occur in perfectly normal surfaces, but are of a sufficiently high order of magnitude to stimulate nerve or muscle.

If the idea is correct that chemical stimulation is due to potential differences set up through an unequal rate of diffusion of the

<sup>8</sup> Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1; 1913, li, 288; 1914, lix, 195; *Science*, 1911, xxxiv, 884; 1913, xxxvii, 672. Loeb, *Science*, 1915, xlii, 643.

<sup>9</sup> Beutner, *Z. physik. Chem.*, 1914, lxxxvii, 385; *J. Am. Chem. Soc.*, 1914, xxxvi, 2040, 2045.

"stimulating" salt through the sheath of Schwann, it follows that these microscopic currents must exist inside of the sheath of Schwann at the boundary of individual axis cylinders or their medullary sheaths. Currents arising at the outer surface of the sheath of Schwann would find in the outside salt solution a conductor of electricity so much better than the nerve fiber that practically no current could go through the latter and hence no "stimulation" could occur.

If these hypothetical statements should be correct they would apply with equal force to the explanation of the chemical stimulation of muscle. In the chemical stimulation of muscle Loeb has formerly described a curious phenomenon which is observed only at the beginning of the experiment when the muscle is put into solutions of  $\text{Na}_3$  citrate,  $\text{Na}_2\text{H}$  phosphate,  $\text{Na}_2$  oxalate, and others. It consists in this, that the muscle which has not yet commenced to twitch in these solutions will go into powerful contractions whenever it is taken out of this solution and put into air, oil, or some other non-conductor.<sup>10</sup> This phenomenon would find its explanation in the assumption that in the beginning of the experiment the outer envelope of the muscle becomes unequally saturated or adsorbed by the stimulating salt and thus gives rise to local potential differences. As long as the muscle is in the solution the currents resulting from these potential differences will flow through the outside solution and no twitching occurs. If the muscle is taken out of the solution the currents are forced to flow through the adjacent muscle fibers and hence cause twitching. This phenomenon was inexplicable before the experiments of Loeb and Beutner were made.

While this explanation of chemical stimulation may seem satisfactory, it must not be forgotten that it is hypothetical. It would not, however, contradict the idea that changes in concentration of ions at the surface of the nerve fibril are the cause of electrical stimulation as Nernst's theory assumes; it would only mean that in the so called chemical stimulation of the nerve potential differences due to unequal diffusion in different spots in the nerve may lead to galvanic currents in the nerve.

An alternative explanation would be that the chemical stimu-

<sup>10</sup> Loeb, *Am. J. Physiol.*, 1900-01, iv, 423.

lation of  $\text{Na}_3$  citrates, etc., or  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  upon nerve (or muscle) is due to some chemical or molecular influence of these salts upon the nerve or muscle fiber, provided the latter have a sufficiently high degree of irritability. This latter theory would make it difficult to understand why compounds of such different chemical constitution as  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  and  $\text{Na}_3$  citrate could both stimulate, while a purely electrical theory would find no difficulty in such facts. Lillie has developed a theory whereby the increase in permeability itself is the essential feature in stimulation;<sup>11</sup> which would agree with some of the facts given in this paper but which would leave unexplained the main fact; namely, why only conductors of electricity will cause twitchings of nerve and muscle.

#### SUMMARY OF RESULTS.

1. It is shown that a non-stimulating salt like  $\text{NH}_4\text{Cl}$  can be made a powerful nerve stimulant if for the four hydrogen atoms ethyl groups are substituted.  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  is a more powerful stimulant for the nerve than sodium citrate.

2. There is little difference between the stimulating power of the chloride, hydroxide, and citrate of  $\text{N}(\text{C}_2\text{H}_5)_4$ , showing that in this case the cation and not the anion determines the high stimulating power.

3. This conclusion is supported by the fact that in order to inhibit the stimulating action of the cation  $\text{N}(\text{C}_2\text{H}_5)_4$  through the addition of  $\text{CaCl}_2$  the same high concentration of this latter salt is required as for the inhibition of the stimulating action of sodium citrate.

4. Both  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  and  $\text{Na}_3$  citrate can only stimulate the nerve when the electrical irritability of the latter is at its normal height. If this irritability is only moderately diminished (by putting the nerve for 2 hours into an isotonic sugar solution) neither sodium citrate nor oxalate nor  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  will stimulate the nerve. If, however, the original irritability of the nerve is restored, by bathing the latter in  $\text{NaCl}$ , sodium citrate as well as  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  will stimulate the nerve again.

5. It can be shown that when enough  $\text{Ca}$  is added to a solution of  $\text{N}(\text{C}_2\text{H}_5)_4\text{Cl}$  or  $\text{Na}_3$  citrate to inhibit the stimulation, the irritability of the nerve is not lowered.

<sup>11</sup> Lillie, R. S., *Am. J. Physiol.*, 1911, xxviii, 197.



6. It can be shown that by adding a quantity of Ca not quite high enough to inhibit entirely the effect of the stimulating salts the latent period of stimulation is considerably increased, a fact which harmonizes with the assumption that the inhibiting effect of Ca is due to a prevention or retardation of the diffusion of the stimulating salt into the nerve.

7. All these facts contradict the theory of Mathews that only the anions have a stimulating effect and that Ca (and all the cations) have a depressing effect.

8. Possible explanations of the connection of diffusibility and stimulating action of salts are discussed.

# THE UTILIZATION OF INOSITE IN THE DOG.\*

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## INTRODUCTION.

In connection with the investigations concerning the occurrence and chemical properties of phytin and inosite phosphoric acids<sup>1</sup> and the physiological rôle of these substances, we have undertaken a few experiments to determine the fate of inosite and its utilization in the animal organism. The previous work on phytin in this laboratory<sup>2</sup> concerned itself chiefly with the physiological relation of the phytin phosphorus to the metabolism. In the present paper we deal with the organic radical, inosite, of the phytin molecule.

The wide distribution of inosite in the vegetable and animal kingdoms has led to many investigations concerning the physiological function of this substance in the animal organism. Since inosite was first discovered in muscle by Scherer<sup>3</sup> it has been found in practically all the tissues and fluids of the body<sup>4</sup> and it

\* Read before the Section of Biological Chemistry at the Urbana meeting of the American Chemical Society, April 20, 1916. The experimental work herein reported was carried out in the Institute of Physiology, University College, London.

<sup>1</sup> Anderson, R. J., *J. Biol. Chem.*, 1912, xi, 471; xii, 97, 447; 1912-13, xiii, 311; 1914, xvii, 141, 151, 165, 171; xviii, 425, 441; 1915, xx, 463, 475, 483, 493; *New York Agric. Exp. Station, Technical Bull.*, 19, 21, 22, 25, 1912; 32, 36, 1914; 40, 1915.

<sup>2</sup> Jordan, W. H., Hart, E. B., and Patten, A. J., *Am. J. Physiol.*, 1906, xvi, 268; *New York Agric. Exp. Station, Technical Bull.*, 1, 1906. Rose, A. R., *New York Agric. Exp. Station, Technical Bull.*, 20, 1912.

<sup>3</sup> Scherer, *Ann. Chem.*, 1850, lxxiii, 322.

<sup>4</sup> A review of the literature will be found in Jacobson, P., and Meyer, V., *Lehrbuch organ. Chem.*, 1902, ii, pt. i, 807.

appears to be a normal constituent of the urine.<sup>5</sup> In the vegetable kingdom, inosite is also widely distributed, occurring free in many plants and, particularly, conjugated with phosphoric acid as phytin or inosite hexaphosphoric acid<sup>6</sup> in seeds, roots, or tubers.

The earlier investigators<sup>7</sup> sought to establish some relation of inosite to the carbohydrates and to the elimination of sugar in diabetes. Külz<sup>8</sup> in an extensive series of experiments examined both normal and diabetic subjects. He found normal human urine practically free from inosite, but from the urine of six normal males after excessive drinking of beer or wine he obtained from 0.4 to 0.9 gm. of inosite. After feeding from 30 to 50 gm. of inosite to normal individuals he was able to recover only from 0.2 to 0.5 gm. of inosite from the urine. In the case of diabetics the same quantity of inosite gave practically the same result and there was no increase in the elimination of sugar. He thought for this reason that inosite might be utilized in the diabetic organism as a source of energy in place of sugar. By experiments on rabbits he also showed that inosite did not give rise to the formation of glycogen.

These studies were later continued by Mayer.<sup>9</sup> His results confirm those reported by Külz concerning the non-formation of glycogen from inosite. After feeding from 2 to 15 gm. of inosite *per os* to rabbits, only small amounts, from 2 to 2.4 per cent of the substance, were recovered from the urine. When it was given subcutaneously, however, he found that from 26 to 51 per cent of the inosite was eliminated unchanged in the urine. In addition to the inosite small quantities of lactic acid<sup>10</sup> were sometimes isolated from the urine after the inosite had been given subcutaneously.

<sup>5</sup> Hoppe-Seyler's Handb. Phys.-u. Path.-Chem. Analyse, Berlin, 7th edition, 1903, 220. Starkenstein, E., *Z. exp. Path. u. Therap.*, 1908-09, v, 378.

<sup>6</sup> A review of literature is given by Rose, A. R., *Biochem. Bull.*, 1912-13, ii, 21.

<sup>7</sup> See Starkenstein,<sup>5</sup> for a review of the literature.

<sup>8</sup> Külz, E., *Sitzungsberichte Ges. z. Beförd. ges. Naturwissen.*, Marburg, 1876; *Beitr. Path. u. Therap. Diabetes Mellitus*, 1874, i.

<sup>9</sup> Mayer, P., *Biochem. Z.*, 1907, ii, 393.

<sup>10</sup> Mayer, *Biochem. Z.*, 1908, ix, 533.

Results very similar to the above were reported about the same time by Starkenstein.<sup>5</sup> About 5 per cent of the inosite given *per os* to rabbits was recovered in the urine. When the substance was injected subcutaneously about 42 per cent and after intravenous injections about 50 per cent of the inosite was eliminated in the urine. This author concludes that inosite is a normal cell constituent; that it is only slightly oxidized in the body because a large percentage of it is eliminated unchanged in the urine after being given in subcutaneous or intravenous injections, and that disappearance of inosite given *per os* is not permissible as proof of its being oxidized by the body since it may be destroyed by bacteria in the intestine.

It is evident from the work which has been done on this subject that inosite given *per os* is either largely destroyed by bacteria in the intestine or else it is stored or oxidized in the body, because very small amounts of it are eliminated in the urine. In the hope of throwing further light upon the fate of inosite in the animal organism we have carried out some respiration experiments with a dog which, in a fasting condition, was fed inosite *per os* just previous to being placed in the respiration apparatus. If inosite were oxidized in the body in the same manner as the carbohydrates or dextrose, for instance, then a rise in the respiratory quotient should be observed. The experiments have shown, however, that there was no appreciable rise in the respiratory quotient after giving 10 gm. of inosite. It is evident, therefore, that inosite is not oxidized in the dog in the manner of dextrose, and if oxidation or other changes take place they do so slowly and in such a way as not to affect the respiratory quotient during the first 2 hours following its ingestion.

It was observed, about 2 or 3 hours after the inosite had been given, that considerable diarrhea set in. Unfortunately it was not possible to separate the urine and the liquid excreta; these were therefore examined together. In one case, however, the liquid feces were voided outside of the metabolism cage and the urine was collected quite clear. When this clear urine was examined it was found to contain but 0.4 gm. of inosite. This shows, as in the case of man reported by Külz<sup>8</sup> and rabbits reported by Mayer<sup>9</sup> and Starkenstein,<sup>5</sup> that but small amounts of inosite are eliminated through the kidneys of the dog. When

the urine was mixed with liquid feces, however, very large quantities of inosite were obtained, and in one case as much as 77 per cent of the amount given was recovered from the mixed excreta.

#### EXPERIMENTAL.

The inosite used in this work was prepared from crude phytin by hydrolyzing this substance with dilute sulfuric acid in an autoclave. It was carefully purified by repeated recrystallization from water. The snow-white product finally obtained was free from ash and melted at 220° (uncorrected).

A small bitch weighing 5,700 gm. was selected for these experiments and trained for use in the respiration apparatus. The animal was kept in a metabolism cage and fed once a day, in the evening, on cooked meat and dog biscuit, but during the period when inosite was given meat was the sole food.

The Benedict respiration apparatus described by Moorhouse, Patterson, and Stephenson<sup>11</sup> was used. The carbon dioxide and oxygen measurements were made as there described, and for particulars we refer to the above paper.

The animal was fed about 5 p.m., and the respiration observations were made about 18 hours afterwards. The quotients in this fasting condition are given in Table I. The figures obtained lie within the limits usually observed under these conditions.

TABLE I.  
*Fasting Respiratory Quotients.*

Date.	Length of period.	CO <sub>2</sub> per minute.	O <sub>2</sub> per minute	R. Q.	Remarks.
	<i>min.</i>	<i>cc.*</i>	<i>cc.</i>		
Feb. 11	42	40.35	54.61	0.739	Usual movements.
" 12	64	44.62	58.65	0.760	Somewhat restless.
" 15	58	42.03	55.72	0.754	" "
" 24	46	35.95	45.65	0.787	Very quiet.

The inosite was given dissolved in about 70 cc. of warm water. This solution was taken at first just as readily as one of glucose,

<sup>11</sup> Moorhouse, V. H. K., Patterson, S. W., and Stephenson, M., *Biochem. J.*, 1915, ix, 176.

but after a while the inosite solution was absolutely refused and it had to be given by the stomach tube. A few minutes after the inosite had been given the animal was placed in the respiration apparatus, and the observation periods varied from  $1\frac{1}{2}$  to about 2 hours. It will be noticed by referring to the figures in Table II that the respiratory quotients during these periods are slightly

TABLE II.  
*Respiratory Quotients after Giving 10 Gm. of Inosite.*

Date	Length of period.	CO <sub>2</sub> per minute	O <sub>2</sub> per minute	R. Q.	Remarks
	min.	cc.	cc.		
Feb. 11'	90	45 18	57 73	0 782	Restless.
" 12	126	44 72	56 50	0 791	"
" 15	123	44 13	56 50	0 780	"
" 17	111	43 38	58 08	0.747	"

higher than those in the fasting condition (Table I). The difference is very slight, however, and it is doubtful if the small rise in the respiratory quotient is due to oxidation of the inosite. The animal showed signs of discomfort during the inosite periods and was more or less restless, consequently the oxygen measurement was more difficult. Diarrhea usually set in a short time after the animal was removed from the respiration apparatus.

For comparison with the quotients tabulated above some figures after feeding glucose are given in Table III.

TABLE III.  
*Respiratory Quotients after Feeding Glucose.*

Date.	Glucose	Length of period	CO <sub>2</sub> per minute	O <sub>2</sub> per minute	R Q	Remarks
	gm.	min	cc	cc		
Feb. 9	20	63	59 77	60 84	0 982	Restless.
" 18	10	78	65 20	66 02	0 989	"

In Table IV are given the amounts of inosite<sup>12</sup> recovered from the urine and liquid feces. In this connection it must be stated that only the liquid portion of the excreta which collected in the bottle underneath the metabolism cage was analyzed for inosite.

<sup>12</sup> The inosite was isolated by the method of P. Mayer.<sup>3</sup>

TABLE IV.

*Inosite Recovered from Urine and Liquid Feces.*

Experiment.	Inosite given.	Inosite recovered.		Remarks
	gm.	gm.	per cent	
1	10	0.40	4.0	Urine clear.
2	10	4.00	40.0	" plus feces.
3	10	1.27	12.7	" " "
4	10	2.25	22.5	" " "
5	10	6.30	63.0	" " "
6	10	7.70	77.0	" " "
1st day } 2nd " }	None.	0.20		" clear.
3rd "	"	Trace.		" "

The variation in the amounts of inosite recovered in the first four experiments is due no doubt to the fact that in these periods the dog was used for the respiration observations and shortly after being removed from the respiration apparatus diarrhea set in and these liquid stools were lost. During the fifth and sixth experiments, however, the dog was kept in the metabolism cage the whole time and all the liquid feces and the urine were collected together.

After the sixth experiment the clear urine was collected for the next 3 days, and analyzed for inosite. By referring to the table it will be noticed that the combined urine of the 1st and 2nd days contained only 0.2 gm. of inosite while on the 3rd day a mere trace was obtained. This shows that practically all the inosite given is eliminated within 24 hours; evidently, therefore, either none or very little of it can be stored in the body.

Since the clear urine (Table IV) contained little and the urine plus liquid feces contained much inosite, it would seem that by far the greater portion of the substance was not absorbed but that it was eliminated through the bowel. In order to determine to what extent inosite is absorbed in the intestine the following experiment was made. A section about 2 feet long of the small intestine of a dog was exposed<sup>13</sup> and washed out with physiological salt solution. One end was ligatured, a solution of 3 gm. of inosite in 50 cc. of warm water was introduced, and the other

The operation was performed by Professor E. H. Starling.

end was also ligatured. The whole was replaced in the abdominal cavity, the incision loosely closed, and the animal kept under chloroform for 2 hours. On removing the section of the bowel it was quite distended and contained 90 cc. of liquid. From this liquid 2.95 gm. of inosite were isolated; *i.e.*, the substance was recovered practically quantitatively.

#### CONCLUSIONS.

Inosite is not utilized to any extent by the dog. It is not stored or oxidized in the body but the greater portion of it is excreted unchanged. When given at the rate of 2 gm. per kg. of body weight it does not cause a rise in the respiratory quotient.

As much as 77 per cent of the amount given may be recovered from the excreta.

Inosite is absorbed very slowly from the intestine, hence it causes a more or less severe diarrhea. As a result it is largely eliminated with the feces and only a small portion is excreted by the kidneys.

The author acknowledges his indebtedness to Professor E. H. Starling for help and advice in carrying out the above experiments, and to Dr. V. H. K. Moorhouse for assistance in using the respiration apparatus.



have been reported by Mayer<sup>3</sup> and Starkenstein<sup>4</sup> who both used rabbits as subjects.

Of the previous investigations concerning the physiological rôle of inosite in man those of Külz<sup>2</sup> are probably the most thorough; he had an unusually large quantity of inosite at his disposal for experimental purposes, having prepared about a pound of it from green beans. The high price and the small available quantities of inosite have presented difficulties in carrying out investigations with this substance. For the present work we prepared several hundred grams of pure inosite as described on page 394.

Our results confirm those of previous investigators that inosite given *per os* disappears and only small quantities are eliminated in the urine. Külz<sup>2</sup> recovered only about 0.9 per cent of the inosite given from the urine of human subjects. Our figures, however, are about ten times greater; *i.e.*, we recovered from the urine nearly 9 per cent of the ingested inosite. Careful examination of the feces was made but we failed to obtain a trace of inosite. Consequently the urine is the only channel of elimination of inosite in man.

In what manner the balance, about 91 per cent, of the inosite is utilized or destroyed is uncertain. It has been shown by the investigations of Meillère<sup>5</sup> and also by Starkenstein,<sup>4</sup> that inosite is destroyed by the colon bacillus. It has also been shown by Hilger<sup>6</sup> and Vohl<sup>7</sup> that inosite is transformed into lactic acid by the bacteria found in putrid cheese. Experiments by Starkenstein<sup>4</sup> indicate that inosite is destroyed by autolytic ferments of muscles and the liver with the simultaneous increase of lactic acid.

Our results do not show whether the inosite was destroyed by bacteria in the intestine or underwent oxidation in the body. If the inosite was changed into lactic acid it is evident that only traces of it were excreted as such, because there was no appreciable increase of ammonia in the urine (Table III). At present we can only record the fact that of the inosite given only about 9 per cent escaped destruction and was recovered from the urine.

<sup>3</sup> Mayer, P., *Biochem. Z.*, 1907, ii, 393.

<sup>4</sup> Starkenstein, E., *Z. exp. Path. u. Therap.*, 1905-09, v, 378.

<sup>5</sup> Meillère, G., *Compt. rend. Soc. biol.*, 1907, lxii, 1096.

<sup>6</sup> Hilger, *Ann. Chem.*, 1871, clx, 333.

<sup>7</sup> Vohl, H., *Ber. chem. Ges.*, 1876, ix, 984.

It is evident from the data presented in the experimental part that, except for the increased excretion of creatinine in the after-period, the ingestion of inosite at the rate of about 0.5 gm. per kg. of body weight has no appreciable influence upon the metabolism of man.

#### EXPERIMENTAL.

To determine the channels of elimination of inosite in man we carried out the following preliminary experiment. One of us (B.) received 30 gm. of inosite dissolved in water in three equal portions during the day. The following morning a solution of 10 gm. of inosite was given. About 4 hours after the first 10 gm. of inosite were taken a very loose watery stool was passed and during the 24 hours there were three more very watery stools. The second day the stools were almost of normal consistency. During the first and second days the subject complained of an uneasy or uncomfortable feeling and of a peculiar sensation in the chest and abdominal muscles. This feeling disappeared towards the end of the 3rd day and on the 4th day he felt normal again.

The urine and feces were collected in 24 hour periods and examined for inosite. For the isolation of the inosite we used the method of Mayer<sup>3</sup> which is briefly as follows: The urine was evaporated to about one-fourth of its volume and precipitated with excess of lead acetate. After standing a short while the precipitate was filtered off on a Buchner funnel and washed with water. The filtrate was heated to boiling and precipitated with excess of basic lead acetate and finally rendered strongly alkaline with concentrated ammonia. After standing for 24 hours the precipitate was filtered on a Buchner funnel and washed with water. It was then suspended in water and decomposed with hydrogen sulfide. After removing the lead sulfide, the filtrate was evaporated on the water bath almost to dryness. The residue was taken up in a little hot water, decolorized with animal charcoal, again evaporated to small bulk, transferred to an Erlenmeyer flask, and brought to crystallization by the addition of about ten volumes of absolute alcohol. After the greater portion of the inosite had crystallized out ether was added until the solution turned cloudy and the whole was allowed to stand in

the ice box over night. The crystals were then filtered off, washed with alcohol and ether, dried in the air, and weighed.

The feces were examined as follows: The fresh material was stirred up with water until of uniform consistency. Lead acetate was then added and after standing for some time it was filtered and washed. The filtrate was evaporated, precipitated with basic lead acetate, etc., as mentioned above.

The amount of inosite recovered in the excreta is given in Table I.

TABLE I.

Day	Inosite given.	Inosite recovered from the urine.		Inosite recovered from the feces
	gm.	gm.	per cent	
1	30	2.6	8.66	None.
2	10	1.1	11.00	"
3	None.	Trace.		"

The results show that in man a small quantity of the ingested inosite is eliminated in the urine and that the feces contain none; also that it is either absorbed and oxidized in the body or else destroyed in the intestine, and that the excretion of unchanged inosite is complete in 24 hours, because on the 3rd day the urine contained only a trace of the substance. We find that the quantity of inosite excreted by the kidneys is about ten times greater than found by Külz. This is probably due to the improved method of isolating the substance.

In a second more extensive experiment we have sought to discover if the ingestion of inosite produced any change or disturbance in the metabolism. For this purpose we selected a period of 8 days during which the food of the subject (B.) was carefully regulated. Unfortunately we failed to maintain any control of the food intake during the days following the actual experiment or after-period. The results are vitiated also by the fact that the period over which our experiment extended was not of sufficient length to bring the subject to a condition of nitrogen equilibrium and that we analyzed only one sample of each article of food. Since it was a liberal and mixed diet consisting of meat, eggs, milk, potatoes, bread, etc., it is probable that the actual intake during the whole period was different from what we figured from the result of one analysis. Despite these drawbacks we feel justi-

fied in publishing briefly the results obtained because these results are negative; *i.e.*, our observations have failed to reveal any noteworthy or striking change in the general metabolism as a result of the ingestion of inosite.

During this second experiment which was begun a few months after the one reported above, we began the inosite period by giving only 15 gm. of the substance the 1st day. On the 2nd and subsequent days 30 gm. of inosite were given. In this way the uncomfortable diarrhea was avoided. The stools were more frequent and softer than usual, but towards the end of the period they were nearly of normal consistency. There were no feelings of discomfort, in fact the subject felt practically normal throughout the experiment, performing his laboratory work in the usual manner.

The total nitrogen and phosphorus balance, with the caution mentioned above, is given in Table II. The difficulty of bringing man to a nitrogen equilibrium and the fact that the diet gave a nitrogen intake quite a little below the usual average daily intake of the subject makes the minus nitrogen and phosphorus balance have no special significance.

TABLE II.  
*Total Nitrogen and Phosphorus Balance.*

Day.	Preliminary period.				Inosite period.			
	1	2	3	4	5	6	7	8
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Nitrogen intake...	15.254	15 483	11 781	12 131	12 880	11 355	11 151	11 249
Nitrogen excretion	15.018	13 602	14 233	12 667	13 943	11 682	13 147	11 821
Phosphorus intake ...	1 832	1 708	1 362	1 384	1 592	1 318	1 323	1 330
Phosphorus excretion.	2.1267	1 6626	1 8688	1 5634	1 8469	1 4029	1 5119	1 4680

		gm.		gm.
Total	nitrogen intake, preliminary period,	54 649;	inosite period,	46 635
"	" excretion, " "	55 520;	" "	50 593
	Minus nitrogen balance,	0 871;		3 958
"	phosphorus intake, preliminary period,	6 286;	inosite period,	5 563
"	" excretion, " "	7 2215;	" "	6 2297
	Minus phosphorus balance,	0 9355;	" "	0 6657

Figures covering the elimination of nitrogen in the urine are tabulated in Table III. There was a considerable rise in the creatinine output which began on the last day of the inosite period and extended through the whole of the after-period, the first 2 days of the after-period showing a marked increase in creatinine elimination. During the after-period the subject ate the same diet as during the rest of the experiment but the food was not accurately weighed. In view of this fact, the increased creatinine output during the after-period would seem to be an important observation and a matter worthy of further study.

The uric acid, creatinine, and ammonia were determined by the Folin methods.

TABLE III.  
*Analysis of the Urine. Nitrogen Elimination.*  
*Preliminary Period.*

Day . . . . .	1	2	3	4
Volume of urine, cc..	1,420	1,480	1,170	710
Specific gravity...	1.019	1.015	1.020	1.028
Total nitrogen, gm..	12.908	12.802	12.273	11.204
Uric acid, gm..	0.636	0.658	0.585	0.546
Creatinine, gm..	1.198	1.199	1.050	1.150
Ammonia nitrogen, gm.	0.965	0.864	0.959	0.899

*Inosite Period.*

Day . . . . .	5	6	7	8
Volume of urine, cc..	770	810	1,450	900
Specific gravity.	1.026	1.026	1.016	1.022
Total nitrogen, gm..	11.504	9.914	11.296	9.234
Uric acid, gm..	0.550	0.463	0.520	0.692
Creatinine, gm..	1.299	1.010	1.068	1.448
Ammonia nitrogen, gm..	1.089	0.868	1.125	0.918

*After-Period.*

Day . . . . .	9	10	11	12
Volume of urine, cc..	1,175	1,390	1,550	1,070
Specific gravity. . . . .	1.022	1.022	1.016	1.023
Total nitrogen, gm... .	12.009	13.232	8.680	8.988
Uric acid, gm.. . . .	0.628	0.731	0.620	0.767
Creatinine, gm... . .	1.729	2.183	1.141	1.332
Ammonia nitrogen, gm... .	0.940	0.970	0.639	0.683

The phosphorus elimination in the urine is recorded in Table IV. The total phosphorus was determined after destroying the organic matter by the Neumann method. The inorganic phosphorus is that quantity which was directly precipitated by ammonium molybdate after acidifying the urine with nitric acid and adding ammonium nitrate. The organic phosphorus was found by difference. It will be noticed by referring to the table that the organic phosphorus varied from 0 to 0.0178 gm. with an average of 0.0102 gm. per day. This agrees with the average quantity of organic phosphorus usually found in normal urine.<sup>8</sup>

TABLE IV  
*Elimination of Phosphorus in the Urine.*  
*Preliminary Period.*

Day.	1		2		3		4	
Volume of urine, cc	1,420		1,480		1,170		710	
	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.
Total phosphorus	0 0835	1 1857	0 0843	1 2476	0 0946	1 1068	0 1433	1 0174
Inorganic phosphorus...	0 0826	1 1729	0 0831	1 2298	0 0946	1 1068	0 1413	1 0032
Organic phosphorus.	0 0009	0 0128	0 0012	0 0178	0	0	0 0020	0 0142

*Inosite Period.*

Day.	5		6		7		8	
Volume of urine, cc	770		810		1,450		900	
	per cent	gm	per cent	gm	per cent	gm	per cent	gm.
Total phosphorus	0 1370	1 0549	0 1174	0 9509	0 0742	1 0759	0 1030	0 9270
Inorganic phosphorus.....	0 1358	1 0456	0 1165	0 9436	0 0738	1 0701	0 1014	0 9126
Organic phosphorus.	0 0012	0 0093	0 0009	0 0073	0 0004	0 0058	0 0016	0 0144

The amount of nitrogen and phosphorus excreted in the feces is given in Table V. A notable rise in the nitrogen excretion is observed during the inosite period. This was no doubt due to the more frequent stools and consequent less complete absorption in the intestine. Since it was shown in the preliminary experi-

<sup>8</sup> For a review of the literature see Forbes, E. B., and Keith, M. H., *Ohio Agric. Exp. Station, Technical Bull.*, 5, 1914, 190.

TABLE V.

*Nitrogen and Phosphorus Excretion in the Feces.*

Day.	Preliminary period.				Inosite period.			
	1	2	3	4	5	6	7	8
Nitrogen, gm.....	2.110	0.800	1.960	1.463	2.439	1.768	1.851	2.587
Phosphorus, gm.....	0.941	0.415	0.762	0.546	0.792	0.452	0.436	0.541

ment that inosite was eliminated only in the urine the feces were not examined this time for this substance.

In Table VI the daily intake of inosite and the quantities recovered from the urine are recorded. The same method of isolation was used as before. The inosite was excreted in about the same proportion as in the preliminary experiment. The total intake amounted to 105 gm. of inosite and only about 9 gm. were recovered. The balance, 96 gm., was either oxidized in the body or destroyed by bacteria in the intestine. Evidently the inosite could not have been stored in the body because the elimination ceases with the intake.

TABLE VI.

*Inosite Intake and Excretion.*

Day.	5	6	7	8	9	10	11	12
Volume of urine, cc....	770	810	1,450	900	1,175	1,390	1,550	1,070
Inosite intake, gm.....	15	30	30	30	None.	None.	None.	None.
Inosite excretion, gm....	0.38	2.5	3.19	2.5	0.47	Trace.	Trace.	Trace.

Total intake, 105 gm. of inosite.

Inosite recovered, 9.04 gm. or 8.6 per cent.

## SUMMARY.

When inosite is taken at the rate of about 0.5 gm. per kg. of body weight per day it produces some diarrhea at first or frequent soft stools. After a few days the stools, although more frequent than usual are nearly of normal consistency.

Except for the increased excretion of creatinine in the after-period, for which we can now offer no explanation, we find that the ingestion of inosite has no marked or appreciable effect upon the general metabolism of man.

About 9 per cent of the inosite taken *per os* is eliminated unchanged in the urine, but none in the feces. In what manner the balance, or about 91 per cent, of the inosite is utilized we have not been able to determine.





# THE NATURE OF THE DISEASE DUE TO THE EXCLUSIVE DIET OF OATS IN GUINEA PIGS AND RABBITS.

By CASIMIR FUNK

(From the Huntington Fund, Loomis Laboratory, and the Harriman Research Laboratory, New York )

(Received for publication, May 9, 1916 )

The subject of the present paper was suggested by the investigations of Morgen and Beger,<sup>1</sup> who fed rabbits on a diet composed wholly of oats. Their results coincided with those obtained by other authors,<sup>2</sup> who also were unable to keep these animals alive for more than a few months. To overcome this they added certain inorganic salts to the diet. Sodium chloride was found to stimulate the food intake, and calcium carbonate to prevent loss in weight, but these failed to decrease the mortality. They concluded that the disease which developed as the result of an exclusive diet of oats was due to acidosis, and tried to prove this by experiments with sodium bicarbonate. When this salt was added to the oats the animals remained in apparently good condition and with one exception lived for more than half a year, when the experiment was discontinued.

Experiments similar to those described by Morgen and Beger have previously been reported. Lunin<sup>3</sup> fed mice on a mixture of casein, fat, and sugar, and found that when sodium carbonate was added to neutralize the sulfuric acid formed from protein the mice lived longer. Weiser<sup>4</sup> fed growing pigs on corn, and the chemical analysis of urine and feces showed a positive nitrogen, but a negative phosphorus and calcium balance. When calcium carbonate was added the latter balances became positive. As opposed to this, Schaumann<sup>5</sup> has found that rabbits are unable to live on corn even upon the addition of calcium carbonate, but do well when dried yeast (5 per cent) is added. Laqueur<sup>6</sup> has shown the beneficial effect of sodium chloride in starving rabbits.

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<sup>1</sup> Morgen, A., and Beger, C., *Z. physiol. Chem.*, 1915, *xiv*, 324.

<sup>2</sup> Holst, A., and Frolich, T., *J. Hyg.*, 1907, *vii*, 634.

<sup>3</sup> Lunin, N., *Z. physiol. Chem.*, 1881, *v*, 31.

<sup>4</sup> Weiser, S., *Biochem. Z.*, 1912, *xiv*, 279.

<sup>5</sup> Schaumann, H., *Arch. Schiffs- u. Tropenhyg.*, 1914, *xviii*, 363.

<sup>6</sup> Laqueur, E., *Résumé des comm. et démonstr.*, IX Congr. Physiol., Gromingen, 1913.

The question arises whether the change that occurs in guinea pigs and rabbits fed on a diet of oats can be considered as scurvy, notwithstanding its similarity to human scurvy. Judging by the inactivity of the known antiscorbutics in the disease described, I have already<sup>7</sup> expressed some doubt as to its nature, as the symptoms of starvation greatly complicate the diagnosis.

The following investigations were undertaken to study the effect of feeding oats to rabbits, guinea pigs, and also to rats, with special reference to the effect of the addition of sodium bicarbonate and the action of antiscorbutics. The conclusions were in accordance with those of Morgen and Beger that the addition of sodium bicarbonate when given in small quantities has a marked beneficial effect in rabbits. This result, together with the inactivity of a vitamine addition in the form of dried yeast, and the failure of the known antiscorbutics, indicates that this condition is probably due to acidosis. In guinea pigs the result was different, as the sodium bicarbonate was found to be without effect and the addition of antiscorbutics a marked failure, making the diagnosis of scurvy uncertain. It was also found<sup>8</sup> that the condition described as scurvy in animals is not suitable for testing chemical fractions obtained from the antiscorbutics, but we do not wish to generalize this statement as we have had no experience with scurvy in monkeys (Hart and Lessing<sup>8</sup>). Whether scurvy in rats will prove a success in this respect is at present under investigation in this laboratory.

The beneficial action of sodium bicarbonate in rabbits fed on oats does not indicate that scurvy is a deficiency disease, since when fed to pigeons, oats are found to contain a sufficient amount of beri-beri vitamine to prevent the outbreak of beri-beri. It even seems possible that rabbits are able to prepare the scurvy vitamine from the beri-beri vitamine present in oats when sodium bicarbonate is supplied.

In the original experiments of Morgen and Beger rabbits were fed oats with sodium bicarbonate added to the extent of 1 per cent, and occasionally other salts were given. In my first experiment the sodium bicarbonate (1 gm.) was introduced by means

<sup>7</sup> Funk, C., *Die Vitamine*, Wiesbaden, 1914.

<sup>8</sup> Hart, C., and Lessing, O., *Der Skorbut der kleinen Kinder*, Stuttgart, 1913.

of a stomach tube; in another series of experiments an equivalent amount of hydrochloric acid was given  $\frac{1}{2}$  hour after the bicarbonate in order to test whether the presence of sodium bicarbonate would not be a sufficient stimulation. Care was taken that the quantity of liquid given was identical in both cases. The result of this experiment is given in Table I, and although sodium bicarbonate stimulated the appetite, it had no life-prolonging action. A possible reason for this might be that the salt was given in too large quantity, 1 gm. at a time having been administered. The food was weighed back every day, but averages will be given for 3 weeks only. The dead animals showed marked scorbutic changes.

TABLE I.

		Weight.		Food intake.		Death after
		Initial.	Final.	Initial.	Final	
		gm.	gm.	gm.	gm.	days
Controls on oats..	I	1,155	775	35	23	49
	II	1,566	880	29	19	70
	III	1,305	884	40	22	57
	IV	1,311	1,035	44	28	28
Oats and NaHCO <sub>3</sub>	I	1,480	1,232	65	33	64
	II	1,387	1,112	50	40	37
	III	1,140	998	41	28	49
	IV	1,276	1,592	65	54	52
Oats, NaHCO <sub>3</sub> , and HCl.....	I	1,623	1,194	53	26	58
	II	1,380	1,340	38	38	27
	III	1,470	1,082	40	36	35
	IV	1,535	1,445	59	59	36

In the next experiment the sodium bicarbonate was added to the extent of 1 per cent of the diet. The method again differed slightly from that used by Morgen and Beger, as the salt was given continuously instead of in periods, and this possibly accounts

## CORRECTION.

On page 410, Vol. xxv, No. 3, July, 1916, the twenty-seventh line of text should read *is not an argument against scurvy being a deficiency disease.*

The question arises whether the change that occurs in guinea pigs and rabbits fed on a diet of oats can be considered as scurvy, notwithstanding its similarity to human scurvy. Judging by the inactivity of the known antiscorbutics in the disease described, I have already<sup>7</sup> expressed some doubt as to its nature, as the symptoms of starvation greatly complicate the diagnosis.

The following investigations were undertaken to study the effect of feeding oats to rabbits, guinea pigs, and also to rats, with special reference to the effect of the addition of sodium bicarbonate and the action of antiscorbutics. The conclusions were in accordance with those of Morgen and Beger that the addition of sodium bicarbonate when given in small quantities has a marked beneficial effect in rabbits. This result, together with the inactivity of a vitamine addition in the form of dried yeast, and the failure of the known antiscorbutics, indicates that this condition is probably due to acidosis. In guinea pigs the result was different, as the sodium bicarbonate was found to be without effect and the addition of antiscorbutics a marked failure, making the diagnosis of scurvy uncertain. It was also found<sup>8</sup> that the condition described as scurvy in animals is not suitable for testing chemical fractions obtained from the antiscorbutics, but we do not wish to generalize this statement as we have had no experience with scurvy in monkeys (Hart and Lessing<sup>8</sup>). Whether scurvy in rats will prove a success in this respect is at present under investigation in this laboratory.

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TABLE I

		Weight		Food intake		Death after
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		gm	gm.	gm	gm.	days
Controls on oats	I	1,155	775	35	23	49
	II	1,566	880	29	19	70
	III	1,305	884	40	22	57
	IV	1,311	1,035	44	28	28
Oats and NaHCO <sub>3</sub>	I	1,480	1,232	65	33	64
	II	1,387	1,112	50	40	37
	III	1,140	998	41	28	49
	IV	1,276	1,592	65	54	52
Oats, NaHCO <sub>3</sub> , and HCl.	I	1,623	1,194	53	26	58
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In the next experiment the sodium bicarbonate was added to the extent of 1 per cent of the diet. The method again differed slightly from that used by Morgen and Beger, as the salt was given continuously instead of in periods, and this possibly accounts for the result obtained. It was found that this salt increased the appetite, but two out of four animals died showing scorbutic changes (Nos. II and III). The first rabbit was killed after 119 days and carefully examined, but no pathological changes were found except a slight fragility of the bones and an enlarged right heart.

TABLE II.

		Weight.		Food intake.		Death after
		Initial.	Final.	Initial.	Final.	
		gm.	gm.	gm.	gm.	days
Oats and $\text{NaHCO}_3$ . . .	I	1,540	1,142	76	29	119
	II	1,620	1,088	67	22	107
	III	1,383	867	55	19	100
	IV	1,730	1,493	66	36	Alive after 123 days.

The final food intake was found to be much smaller than the initial one, indicating perhaps that all animals would eventually die. This again does not corroborate Morgen and Beger's results. That rabbits show great variability when kept on oats is demonstrated in Table III. In this experiment two rabbits were kept on oats alone much longer than the controls of the first experiment. The first of these rabbits was pregnant and gave birth to eight apparently normal young after 40 days on a diet of oats. This animal lived during 95 days on this diet and was in good condition when the experiment was discontinued, showing only a moderate loss in weight.

TABLE III.

		Weight.		Duration.
		Initial.	Final.	
		gm.	gm.	days
Oats.	I (Pregnant.)	3,690	3,306	95
	II	2,036	1,500	76

*The Action of Yeast and Various Antiscorbutics in Rabbits Fed on Oats.*

In this experiment four rabbits were given oats with 1 per cent yeast for 12 days, then 10 to 20 cc. of potato juice for 6 days, and finally yeast and potato juice together. The juice was prepared by grinding fresh potatoes and filtering the pulp with suction without diluting it. The figures under "Food intake" are successive weekly averages. The experiment, however, was discontinued as the food intake was poor in spite of the additions.

TABLE IV.

		Weight.		Average food intake.	Duration.	Result.
		Initial.	Final.			
		gm.	gm.	gm.	days	
Oats, yeast, and potato juice.	I	1,470	1,251	62, 52, 26, 12	35	Died.
	II	1,260	1,143	63, 59, 26, 8	35	Discontinued.
	III	1,258	1,024	47, 34, 21, 14	35	Died.
	IV	1,265	1,342	81, 62, 25, 35	35	Discontinued.

Rabbits I and III showed scorbutic changes at autopsy. The experiment with yeast was repeated on several other rabbits with identical results.

Besides yeast and potato juice a series of experiments were undertaken with germinated oats, which, according to Fürst<sup>9</sup> prevent the outbreak of scurvy in guinea pigs. Apparently the soaked seeds were not a suitable diet, as the animals died in a short time.

#### *Experiments on Guinea Pigs.*

The experiment in which sodium bicarbonate was given to rabbits was repeated with guinea pigs, the salt being mixed with

TABLE V.

		Weight.		Food intake.		Death from scurvy after
		Initial.	Final.	Initial.	Final.	
		gm.	gm.			days
Oats.	I	388	239	Food intake steadily decreasing.		30
	II	366	274			24
	III	371	252			22
	IV	392	305			22
				1st week.	2nd week.	
		gm.	gm.	gm.	gm.	
Oats and NaHCO <sub>3</sub> .	I	502	364	19	2	18
	II	539	383	25	24	24
	III	644	468	29	2	17
	IV	525	543	41	22	17

<sup>9</sup> Fürst, V., *Z. Hyg. u. Infektionskrankh.*, 1912, lxxii, 121.



the oats. The result obtained differed from that with rabbits, in that the animals died earlier than the controls. We therefore are not justified in considering that the pathological condition due to the feeding of oats is acidosis.

In Table VI the results of the experiments with various anti-scorbutics on guinea pigs are summarized. Experiments were undertaken with potato juice, milk, lime juice, and with various fractions prepared according to a previous paper.<sup>10</sup> The best preventive action was obtained with milk, but only when large quantities were given.

TABLE VI.

		Weight.		Duration.	Result.
		Initial.	Final.		
		gm.	gm.	days	
Controls on oats.	I	425	315	26	Died; symptoms of scurvy.
	II	475	330	24	" " " "
	III	615	380	16	" " " "
	IV	457	250	27	" " " "
	V	427	250	22	" " " "
	VI	390	230	21	" " " "
	VII	348	207	30	" " " "
	VIII	372	231	24	" " " "
	IX	267	154	27	
	X	261	164	25	
	XI	263	177	22	
	XII	248	190	24	Died; symptoms of scurvy.
	XIII	310	180	24	" " " "

In the last period nothing or very little was eaten. Average duration 24 days. Average loss in weight 37 per cent.

<sup>10</sup> Funk, *Biochem. J.*, 1913, vii, 81.

TABLE VI—*Concluded.*

		Weight.		Duration.	Result.
		Initial	Final.		
Phosphotungstic acid fraction from 15 liters of lime juice made up to 1 liter. 20 cc. daily, and oats.	I	481 640 752 542	325 315 600 290	24 28 20 30	Died of scurvy. " " " " " " Average duration 25 days. Died of scurvy. Average loss in weight 34 per cent.
Phosphotungstic filtrate fraction in amount corresponding to the above, and oats.		880 700 605 435	700 425 470 335	14 12 16 18	Alive. Died of scurvy. " " " " " "
25 cc. potato juice daily and oats.		392 391	238 244	34 36	" " " Loss in weight 38 per cent. Died of scurvy.
25 cc. milk daily and oats.		467 447	292 393	38 50	" " " Alive.
50 cc. milk daily and oats.		395 354	375 440	50 50	" "
Protein-free milk, corresponding to 200 cc. fresh milk, and oats.		438 319 342 415	269 185 247 287	29 38 33 35	Died of scurvy. " " " " " " " " "
Alcoholic extract from 3 liters of milk made to 300 cc., 40 cc. daily, and oats.		449 340	268 282	24 14	" " " " " "
30 gm. milk powder extracted with alcohol added, and oats.		163 201	90 161	25 92	" " " Alive.
The same diet as above but no extract.		142 112	168 90	92 24	" Died of scurvy.

*Rats on Diet of Oats.*

Rats can be maintained on oats and white bread for a long time, although it was found that young rats do not grow on this diet and the addition of sodium bicarbonate had no beneficial action. The failure of young rats to grow on oats coincides with experiments<sup>11</sup> on young chickens that failed to grow on unpolished rice. We even use the diet of oats and white bread to inhibit the growth of rats prior to feeding of the experimental diet. Further details on oats in relation to growth will be published later. Rats which are fed on oats autoclaved at 15 pounds' pressure die quickly, as is shown in Table VII.

TABLE VII.

Weight.		Duration.
Initial.	Final.	
gm.	gm.	days
105	66	20
76	59	6
103	56	9
140	80	21

## SUMMARY.

The symptoms that develop in rabbits fed on oats are due possibly to acidosis and not to scurvy, judging from the beneficial effect of sodium bicarbonate and the ineffectiveness of the antiscorbutics. Guinea pigs on the same diet are not influenced by the alkali and respond so slightly to the action of antiscorbutics that the identity of this condition with human scurvy seems doubtful. Rats can live on oats for a considerable time, but not on autoclaved oats; young rats, however, fail to grow on this diet.

<sup>11</sup> Funk, *Proc. Roy. Soc. Med.*, 1913, vii, 9; *Z. physiol. Chem.*, 1913, lxxxviii, 352.

## THE COMPOSITION AND PHYSIOLOGICAL ACTIVITY OF THE PITUITARY BODY. II.

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(Received for publication, May 5, 1916 )

### *Comparison of Activity of Adult and Infant Glands.*

In a previous communication<sup>1</sup> it was shown that no distinct seasonal variation exists in the physiological activity and chemical composition of the pituitary body of cattle. Only glands from adult animals were investigated.

The material for the present work was selected from calves 2 to 4 months old, as well as from full grown cattle. None of the calves had been castrated or spayed, while a large majority of the mature animals were steers. Glands available for manufacturing purposes are obtained from the large abattoirs, and the majority of cattle slaughtered for human consumption are steers. With the exception of the sex glands and the thymus, the various organotherapeutic preparations are manufactured from glands obtained from castrated and non-castrated animals indiscriminately.

It has been pointed out that a high percentage of pituitary glands from cattle contain solid colloidal deposits between the anterior and posterior lobes at all four seasons. These deposits, which take the shape of the intralobular space and often expand the gland to the point of deformity, were found both in large and small glands, and no relation could be established between the size of the colloidal substance and of the gland. A number of pituitary bodies from young calves contained a light colored, opalescent, viscous liquid, but no solid colloidal material. The formation of solid colloids, therefore, is not a normal occurrence

<sup>1</sup> Fenger, F., *J. Biol. Chem.*, 1915, **xxiii**, 283.

during the growth period, but happens in the adult stage. The solid colloidal material is insoluble in all the ordinary solvents and is physiologically inert. It is evident, therefore, that the inspissated colloidal masses consist of the insoluble non-dialyzable portion of the pituitary secretion, which in the course of time has collected in such quantities as to be obstructive and undoubtedly of considerable hindrance to the normal functions of the gland.

The calf glands were collected from approximately 900 healthy, non-castrated calves, 2 to 4 months old, during December, 1915. The pituitaries from full grown cattle, representing many thousand animals, were obtained during the summer and fall of 1915. The method of collecting and preparing the glands for analytical purposes was identical with that described in detail in a previous communication.<sup>1</sup> In Table I are given the weights of the fresh glands and the yield of posterior lobe, also the moisture, the petroleum ether-soluble portion, and the yield of desiccated fat-free material. In the desiccated, fat-free, powdered material the moisture, ash, and total phosphoric acid were determined. Only the calf glands were submitted to chemical analysis, as various lots of the glands from mature animals have been analyzed and reported in a previous paper.<sup>1</sup>

TABLE I.

*Lobes from Calves Collected December, 1915.*

No. of glands.....	875	
Weight, gm.		
Average.....	0.7	
Maximum.....	1.1	
Minimum.....	0.4	
Yield of posterior lobe from whole glands, per cent..	12.6	
	Anterior.	Posterior.
	per cent	per cent
Moisture in fresh tissue.....	77.6	77.8
Petroleum ether-soluble.....	1.3	2.6
Desiccated fat-free material.....	21.1	19.6
Moisture.....	4.50	6.05
Ash.....	4.80	5.89
P <sub>2</sub> O <sub>5</sub> .....	2.68	2.63

These figures do not differ materially from those obtained from full grown cattle, except that the phosphoric acid is slightly higher, both in the anterior and posterior lobes of the calf glands.

The petroleum ether-soluble portion of the posterior lobe from pituitary bodies is a yellowish brown, waxy substance resembling crude phosphatides as obtained from brain tissue by the same solvent. It forms emulsions with water readily, but possesses no uterine-contracting power. A sample contained 4.74 per cent of  $P_2O_5$ , equivalent to 53.3 per cent of lecithin.

The physiological activity of the posterior lobe, both from calf and cattle glands was determined by the isolated uterus method as outlined by Roth.<sup>2</sup> The various pituitary solutions were made to represent a 5 per cent solution of the fresh posterior lobes. The glands were finely minced, macerated with slightly acidulated isotonic salt solution, heated to boiling, and filtered. The clear liquid was filled into ampules and sterilized. By mincing the glands exceedingly fine and by improved methods of extraction and filtration, solutions were produced which far surpassed in activity those prepared in this laboratory and reported a year ago.<sup>1</sup>

The standard was prepared by dissolving 0.1 gm. of  $\beta$ -imidazolethylamine hydrochloride (hereafter referred to as  $\beta$  I) in 100 cc. of distilled water. This liquid was poured into ampules and sterilized at the same time and temperature as the lots of pituitary liquid. The standard as well as the various pituitary liquids were made in duplicate. A 1:20,000,000 dilution of the standard in Locke's solution was maintained throughout the experiments. Various dilutions of the pituitary liquid were tried until the exact strength was found which matched the contraction produced by the standard. It may be mentioned here that the dilution of the standard in Locke's solution keeps well throughout the day, but the dilutions of pituitary liquid must be tested soon after they are made up as Locke's solution has a deteriorating effect on the uterine-contracting active principle, and solutions lose considerable strength on standing for 1 or 2 hours.

The selection of a suitable uterus is not always an easy matter. Often three or four guinea pigs have to be killed before a satis-

<sup>2</sup> Roth, G. B., *Bull. Hyg. Lab., U. S. P. H.*, No. 100, 1914.

factory strip is obtained. The individual sensitiveness towards pituitary and  $\beta$  I varies considerably. It is, consequently, only by comparing several tracings made on different days that a fairly correct conclusion in regard to the actual strength of a certain solution may be drawn. It would be desirable to obtain a standard which resembles more closely the active principle of the posterior lobe than does  $\beta$  I. In this laboratory a 2.5 cm. strip of the uterine end of one of the horns is employed. The ovarian end is discarded. The attachments are made by running a silk thread through the strip with the aid of a fine needle and then making a loop once around the entire strip and fastening with a knot. This prevents sagging of the uterus during the test and does not interfere with its sensitiveness. The silk attachments are made while the uterus is still in place in the animal. This saves time as well as unnecessary and undesirable handling of the strip. It generally takes from 1 to 1½ hours to adjust the uterus and balance it so that the rhythmical contractions are regulated to the proper size. Concordant and reliable results may then be obtained for 3 or 4 hours. After that time the uterus becomes hypersensitive and the tracings are consequently unreliable. Some uteri are rather slow in getting back to rhythmical contractions after the active solutions have been replaced by the blank, but in all instances this must be done before a fresh test solution is added. In case the guinea pig is light and the uterus thin, both horns may be tied together and the lower attachment fastened well into the body of the uterus.

In the present work determinations were made several times, on different days during the last few months, and it was found that the following dilutions of the original 5 per cent solutions of the posterior lobes were necessary to produce the same contractions as a 1:20,000,000 dilution of the standard.

Posterior lobes from full grown cattle.....	1 : 38,000
“ “ “ young calves.....	1 : 40,000

These figures indicate that the pituitary bodies from young animals are slightly more active than the glands from full grown cattle. This is in accordance with conditions found in other ductless glands, such as the thyroid, thymus, and suprarenals.

*Observations on the Extraction of the Glands.*

In order to determine the quantity of the water-soluble portion of the posterior lobe, 10 gm. portions of the fresh, finely minced lobes were extracted with water containing 0.5 per cent of glacial acetic acid, boiled for 10 minutes, cooled, made up to 100 cc., and filtered. If no acid is used a turbid solution results from colloidal suspension of lecithin and similar products, and it is impossible to filter the liquid clear. The filtrate after evaporation *in vacuo* to constant weight gave 0.691 gm. of total solids. In other words, 6.91 per cent of the fresh posterior lobe is soluble in acidulated water. The dry, acid water-soluble extractives possessed a uterine-contracting power of approximately one-half the strength of the crystalline  $\beta$  I. This residue forms yellow, hygroscopic, amorphous scales, and contains besides the uterine-contracting active principles, considerable amounts of phosphates and a high percentage of water-soluble organic extractives.

The uterine-contracting active principle may also be extracted by means of alcohol, as shown by the following experiments. Some finely minced fresh posterior lobes were extracted with neutral, and others with acidulated methyl or ethyl alcohol, using 100 gm. of glands and 200 cc. of alcohol. The glands were macerated with the solvents, heated to boiling, and filtered. The residues were washed with more solvent and the filtered liquids evaporated to dryness *in vacuo*. The dry residues thus obtained were exceedingly active. The acidulated methyl alcohol extract, for instance, showed a uterine-contracting power somewhat stronger than pure  $\beta$  I. This particular residue was of a yellowish brown color and contained lipoids, organic extractive matters, phosphates, etc. It was only partly soluble in water and it is certain, therefore, that the pure active principle of the posterior lobe must be very much stronger in uterine-contracting power than crystalline  $\beta$  I. The lipoids, although inert themselves, seem to be closely associated with and to exert a protective action upon the active principle. If, for example, the desiccated alcohol extract is redissolved in acidulated water in which the lipoids are insoluble, and the clear filtrate evaporated to dryness, the resulting extract is lower in uterine-contracting power than the original alcoholic extract. The active principle is apparently



susceptible to oxidation from the air. This is shown by the fact that solutions made from the desiccated fat-free gland are less active than those made from the fresh gland when proportionally equal amounts are employed.

#### SUMMARY.

The physiological activity of the posterior lobe of the pituitary body is somewhat higher during the growth period than after maturity. This is analogous to the conditions existing in the thyroid, the thymus, and the suprarenals. The infant gland contains more phosphates both in the anterior and posterior lobes than glands from fully mature animals.

The uterine-contracting active principle of the posterior lobe of the pituitary body is readily extracted from the fresh glands by water and also by neutral and acidulated methyl or ethyl alcohol. The acidulated methyl alcohol extract is more than twice as strong as the water extract and somewhat stronger than pure crystalline  $\beta$ -imidazolylethylamine hydrochloride.

# THE RÔLE OF PSYCHIC AND SENSORY STIMULI IN THE HYPERGLYCEMIA PRODUCED BY LOWERING THE ENVIRONMENTAL TEMPERATURE OF DOGS.

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(Received for publication, May 2, 1916.)

In 1845 Claude Bernard<sup>1</sup> discovered that glucose is present in normal blood. Since this discovery a large number of articles have appeared dealing (1) with methods for the quantitative estimation of the sugar content of the blood of various animals; (2) with the relative distribution of the sugar between the corpuscles and plasma; (3) with the nature of the reducing substances in blood; and (4) with the variations in the amount of sugar in the blood under physiological and pathological as well as under experimental conditions. This voluminous literature has been ably reviewed by Bang,<sup>2</sup> Macleod,<sup>3</sup> and Allen.<sup>4</sup>

Cannon and his collaborators<sup>5</sup> made the discovery that during strong emotion there occurs an increase in the percentage of sugar in the blood of cats synchronous with the appearance of a substance in the blood which inhibits the rhythmical contractions of the intestinal segment. Hirsch and Reinbach<sup>6</sup> subsequently found that a hyperglycemia of considerable degree may be produced if a dog is tied to a frame. These observations made it necessary to reinvestigate the work of earlier investigators with

<sup>1</sup> Bernard, C., *Leçons sur le diabète*, Paris, 1855, 175.

<sup>2</sup> Bang, I., *Der Blutzucker*, Wiesbaden, 1913.

<sup>3</sup> Macleod, J. J. R., *Diabetes: Its Pathological Physiology*, New York, 1913, 23.

<sup>4</sup> Allen, F. M., *Studies concerning Glycosuria and Diabetes*, Cambridge, 1913.

<sup>5</sup> Cannon, W. B., Shohl, A. T., and Wright, W. S., *Am. J. Physiol.*, 1911-12, xxix, 280.

<sup>6</sup> Hirsch, E., and Reinbach, H., *Z. physiol. Chem.*, 1914, xci, 292.



month, so that the animal became thoroughly accustomed to it before the analyses were accepted.

A sample of blood was drawn at 10 a.m. and the room temperature was noted. The windows were then opened, the room was rapidly cooled, and blood was collected at intervals of  $\frac{1}{2}$ , 4, and 6 hours. In some cases the cage was carried to a cold room. The room was then warmed and another sample of blood obtained. In subsequent experiments the animal was exposed to a warm environment for several days, then to a cold one for 4 days, and then again to a warm atmosphere for an equal period of time. Two and occasionally three samples of blood were drawn during the day at 4 and 6 hour intervals. All analyses are averages of duplicates which agree within 2 mg. of glucose in 100 cc. of blood.

*Collecting the Blood.*<sup>13</sup>—Hirsch and Reinbach drew blood in a similar manner. The dog's ear was shaved 1 or 2 days before and cleaned with alcohol and ether. When about to collect the blood the animal was gently lifted from the cage by the man who fed her, placed upon a table, and an incision was made into the edge of the ear with a sharp razor. The first few drops were allowed to flow away and subsequent drops were caught in a wide-mouthed bottle which contained a pinch of potassium oxalate powder. The application of a little pressure with a piece of sterile gauze or a piece of clean paper towel frequently sufficed to stop the flow, and when this was later removed blood again commenced to drip from the incision. In most cases the dog showed no discomfort during the entire procedure. Detailed notes were made as to the conduct of the dog while blood was being drawn and occasionally during the interim.

*Method of Analysis.*—The method used for estimating the sugar content of the blood was that of Lewis and Benedict,<sup>14</sup> the original technique being closely followed. A solution of Kahlbaum's pure *Traubenzucker* was used as a standard. This was restandardized from time to time. By drawing from 2 to 6 cc. of blood each time it was possible to analyze two or three specimens from the same sample and thereby obtain from four to six aliquots from each sample of blood collected. In order to test the accuracy of the method in our hands we analyzed specimens of blood of known sugar content to which definite quantities of the glucose standard had been added. Table I shows that glucose added to blood was recovered almost quantitatively. In view of the fact that but slight changes in blood sugar content were expected it was necessary to show that our margin of technical error was small. Duplicates and triplicates were therefore done on the same sample of blood and, as seen in Table II, our maximal variation is 2 mg. of glucose in 100 cc. of blood.

All colorimetric readings were made by one of us (K). Long<sup>15</sup> calls attention to the tendency of colorimeter prisms to slip down when the

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<sup>13</sup> The method of obtaining the blood was suggested to us by Dr. F. P. Underhill.

<sup>14</sup> Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

<sup>15</sup> Long, J. H., *J. Am. Chem. Soc.*, 1916, xxxviii, 716.

external temperature is high, due to the liquefaction of the wax by means of which the prism is fixed in the collar, thereby introducing an error in reading the colorimeter. We were aware of this danger and therefore read the standard against itself both before and after each set of readings

TABLE I.

*Recovery of Glucose Added to Blood of Known Sugar Content.*

Specimen	Theoretical.	Found	Difference	
	mg		mg	per cent
51	3 365	3 412	0.047	1 37
53	3 287	3 300	0 013	0 39
58	3 159	3 216	0.057	1.7

TABLE II.

*Margin of Error in Duplicates.*

Specimen	A.	B	Specimen	A	B
	mg.	mg.		mg.	mg
64	0 087	0 087	79	0.081	0 083
65	0 094	0 095	84	0 091	0 089
67	0 105	0 104	82	0 096	0 098
76	0 098	0 096	85	0 083	0 083
77	0 097	0 097	86	0 089	0 087
78	0 092	0 091	87	0 084	0 087
				0 091	0 091

In Table III we have recorded the percentage of sugar in the blood, the room and rectal temperature, the day and hour that the blood was drawn; also whether or not it was necessary to make a new incision in order to get a sample of blood. In the last column the conduct of the dog during the collection of the blood is indicated. This table shows that in the absence of sensory and psychic stimulation there is no appreciable change in the percentage of sugar in the blood of a normal dog even when the animal is exposed to a room temperature of 2°C. for 3½ hours and the environmental temperature fluctuates from 2-26°C. On the other hand, if by rubbing the incision the dog be made uncomfortable, considerable increase in the sugar content of the blood may be observed. When the blood sugar had risen to 0.104 per cent a new incision was made and the blood sugar content at

once began to drop in spite of the simultaneous fall in the external temperature, thus indicating clearly that the essential factor is not the external temperature but the emotional state of the ani-

TABLE III

Date.	Specimen.	Glucose.	Temperature		Hour	Source, old or new incision	Conduct
			Room	Rectal			
1916		per cent	°C.	°C.			
Jan. 25	36	0.093	24	38.6	8 30 a.m.	Old.	Quiet.
" 26	37	0.090	14	38.8	2 10 p.m.	"	" unconcerned.
" 26	38	0.090	26	38.6	5 15 "	"	Resisted handling.
" 27	39	0.087	20.5	38.4	1 50 "	"	Quiet.
" 27	40	0.089	8.0	38.7	4 50 "	"	" unconcerned.
" 28	42	0.090	+ 2.0	38.8	12 00 m.	"	(Exposed to cold for 3½ hrs.) Quiet.
Average		0.089					
" 31	44	0.092	15.5	38.7	9 00 a.m.	New	Struggled at first.
" 31	45	0.089	- 4	38.7	10 00 "	"	" "
" 31	46	0.100	0.0	38.7	3 00 p.m.	Old.	Excited by class in adjoining room.
Feb. 1	47	0.091	18	38.6	10 25 a.m.	"	Quiet. (Cage carried to cold room.)
" 1	48	0.093	+ 2	38.7	11 00 "	"	Whined a little.
" 1	49	0.096	- 3	39.2	3 00 p.m.	"	Shivered, otherwise quiet
" 5	51	0.104	2.6	39.1	10 40 a.m.	"	Cut did not bleed freely; rubbed cut with gauze.
" 5	52	0.104	0.0	38.8	3 10 p.m.	"	
" 8	53	0.100	16	38.9	10 30 a.m.	"	Resisted slightly.
" 8	54	0.103	+ 3	39.1	11 25 "	"	
" 8	55	0.104	+ 3.7	38.8	2 45 p.m.	"	Restless.
" 9	56	0.100	+20.0	38.8	9 25 a.m.	New.	Ear appeared tender so a new cut was made.
" 9	57	0.096	4.1	38.9	10 20 "	"	Quiet.
" 9	58	0.094	4.7	38.8	2 40 p.m.	"	Quiet.
" 9	60	0.093	21.2	38.8	10 30 a.m.	Old.	"

mal. However, if a dog is kept in a cold environment for a period of 24 hours or more a hyperglycemia appears which is not to be attributed to sensory stimulation or the emotional state of the animal.

TABLE IV.

*Effect on the Blood Sugar of Long Continued Exposure to Warmth.*

Date.	Specimen.	Sugar.	Temperature.		Duration of exposure.	Source, old or new incision.	Conduct.
			Room.	Rectal.			
1916		per cent	°C.	°C.	hrs.		
Feb. 25	62	0.088	32	38.77	24	New.	Quiet.
" 25	63	0.088	33	38.83	26	Old.	"
" 25	64	0.087	31	38.72	29	New.	"
Mar. 6	78	0.091	25	38.66	43	"	Barked.
" 6	79	0.082	29.5	38.72	49	Old.	"
" 7	80	0.091	25.5	38.44	67	New.	Quiet.
" 7	81	0.091	29.0	38.5	71	Old.	"
" 8	83	0.089	29.2	38.44	93	New.	"
" 8	84	0.090	28.7	38.44	96	Old.	"
" 9	85	0.083	23	38.44	113	New.	
" 9	86	0.089	30	38.61	118	Old.	
" 9	87	0.083	31.5	38.66	120	"	Quiet.

The average percentage of blood sugar is 0.087.

TABLE V.

*Effect on the Blood Sugar of Long Continued Exposure to Cold.*

Date.	Specimen.	Sugar.	Temperature.		Duration of exposure.	Source, old or new incision.	Conduct.
			Room.	Rectal.			
1916		per cent	°C.	°C.	hrs.		
Feb. 26	65	0.095	5.4	38.50	17	Old.	Quiet, shivered.
" 26	67	0.104	4.1	38.55	21	"	" "
Mar. 1	68	0.107	7.0	39.00	48	New.	" "
" 1	69	0.106	7.0	38.88	50	Old.	" no shivering.
" 2	70	0.105	2.2	38.61	68	"	" shivered.
" 2	71	0.110	5.0	38.66	71	"	" "
" 3	72	0.097	7.0	38.66	100	New.	Shivered.
" 3	73	0.102	1.0	38.61	102	Old.	Quiet.
" 3	74	0.101	7.2	38.66	106	"	" no shivering.
" 3	75	0.109	4.0	38.88	108	"	No shivering.
" 4	76	0.097	0.2	38.55	125	"	Quiet, shivered.

The average percentage of blood sugar according to this table for continuous exposure to cold up to 125 hours is 0.102 per cent. The percentage increase in sugar as a result of long continued exposure to cold is 14.2 per cent, above the average obtained at room temperatures varying from 23-33°C., as shown in Table IV.

Weiland<sup>16</sup> found that muscular exercise, which does not produce a rise in body temperature and is not accompanied by respiratory distress, produces a fall in blood sugar, while Lusk<sup>17</sup> has succeeded in freeing the tissues of normal healthy men of their glycogen by immersion in cold baths that caused the individual to shiver. The question might be raised as to whether the increase in the percentage of sugar in the blood is due to cold alone or to shivering incidentally produced. The work of Weiland on the effect of muscular activity on blood sugar would lead one to expect the opposite effect; namely, a fall in blood sugar during shivering. Table V shows that the blood sugar was no higher when the dog was exposed to cold and shivered, than it was when she did not shiver, and in some cases it was even lower. Thus in Experiments 65 and 76, when the dog did shiver, the blood sugar was lower than the average for cold, while in Experiment 69, when the animal did not shiver, it was not lower than in the preceding or subsequent experiments when the dog did shiver. Specimen 72 is comparatively low, although the dog shivered, while Specimens 73 and 74 are much higher than the average at high temperatures, although the animal did not shiver in either case.

In the subsequent experiment the dog was exposed to a room temperature of 17°C. for 5 hours, at the end of which period, that is, at 3.45 p.m., a specimen of blood was drawn, and the blood sugar content found to be 0.085 per cent. The animal was quiet and not shivering. The room was then cooled to a temperature of about 12°C. The atmosphere was chilly and penetrating. The dog was exposed to this environment over night, and the next morning at 9.30 another sample of blood was collected. At this time the dog shivered. Duplicate analyses of this sample gave 0.084 and 0.086 per cent. The conclusion seems justified that shivering does not increase the percentage of glucose in normal dog's blood, but may decrease it.

<sup>16</sup> Weiland, W., *Deutsch. Arch. klin. Med.*, 1908, xcii, 223.

<sup>17</sup> Lusk, G., *Am. J. Physiol.*, 1910-11, xxvii, 427; 1908, xxii, 163.



## CONCLUSIONS.

In the absence of adequate sensory and psychic stimulation the percentage of sugar in the blood of a normal dog did not vary when the animal was exposed to changes in external temperature for brief periods. A definite hyperglycemia followed the exposure of this animal to a low external temperature for a period of 24 hours or longer.

# THE NATURE OF THE ACID-SOLUBLE PHOSPHORUS OF SERUM.

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(Received for publication, May 22, 1916.)

In previous communications<sup>1</sup> the author has shown that phosphorus exists in the serum of dogs and of men in two classes of compounds: (1) phospholipins; (2) a form which is insoluble in the usual organic solvents but is soluble in a mixture of dilute acetic, or hydrochloric, and picric acids. The opinion was advanced that the latter was inorganic phosphate or a form closely related thereto. The question has been further investigated and the results obtained are presented in this paper.

In order to avoid the use of excessively large volumes, the serum was mixed with five volumes (instead of ten, as in previous work) of a 1 per cent solution of acetic acid saturated with picric acid. After standing 30 minutes, this was filtered and aliquots were taken for the following determinations.

A. Two portions of 500 cc. each were concentrated in a Kjeldahl flask, oxidized with sulfuric and nitric acids, and the phosphorus was precipitated as ammonium phosphomolybdate. This was filtered out, dissolved in ammonium hydroxide, the solution neutralized with citric acid, and the phosphate reprecipitated as ammonium magnesium phosphate.

B. Two portions of 500 cc. each were diluted with 300 cc. of water (to prevent subsequent precipitation of large amounts of ammonium picrate<sup>2</sup>), magnesia mixture and ammonium hydroxide were added, and the mixture was allowed to stand over night. The precipitate was then filtered out and washed with dilute

<sup>1</sup> Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 369; 1915, xxi, 29; *Am. J. Med. Sc.*, 1914, cxlvii, 225.

<sup>2</sup> Ammonium picrate, though readily soluble in water, is quite insoluble in solutions of ammonium salts.

ammonium hydroxide, dissolved in cold citric acid, and reprecipitated by the addition of magnesia mixture and ammonium hydroxide.

C. Two portions of 500 cc. each were diluted with 300 cc. of water (to prevent subsequent precipitation of picric acid) and 125 cc. of molybdate solution<sup>3</sup> were added. A yellow precipitate appeared in a few seconds and settled slowly.<sup>4</sup> After standing at room temperature over night, it was filtered out, dissolved in dilute ammonium hydroxide, the solution neutralized with citric acid, and the phosphate reprecipitated with magnesia mixture.

Marriott<sup>5</sup> has reported a method for the determination of inorganic phosphate in small amounts of serum. He found from 1 to 3 mg., calculated as phosphorus, per 100 cc. of normal adult's serum. The inorganic phosphate was precipitated from the diluted serum by means of magnesia mixture. A similar technique was used in this work. Two portions of 100 cc. each of the serum were diluted with 200 cc. of water and treated with magnesia mixture and ammonium hydroxide. After standing over night the precipitate was filtered out, washed, dissolved in cold citric acid solution, and the phosphorus reprecipitated with magnesia mixture and ammonium hydroxide. The first precipitate could not be used directly as it contained considerable foreign material.

The precipitates obtained by any of these procedures were filtered on Gooch crucibles, washed with dilute ammonium hydroxide, dried in a water-jacketed oven, weighed, dried at 105° (Freas oven), weighed, and finally ignited and weighed. This was done to establish the constitution of the precipitate as ammonium magnesium phosphate and also to obtain data on the advisability of the routine weighing of the precipitate without

<sup>3</sup> This was prepared by dissolving 100 gm. of molybdic acid (85 per cent) in a mixture of 155 cc. of concentrated  $\text{NH}_4\text{OH}$  and 300 cc. of  $\text{H}_2\text{O}$ , and adding this to a mixture of 530 cc. of concentrated  $\text{HNO}_3$  and 824 cc. of  $\text{H}_2\text{O}$ .

<sup>4</sup> The precipitate obtained by adding molybdate solution to a phosphate solution containing picric acid appears to be quite different from the usual ammonium phosphomolybdate. It was not analyzed, but one striking difference is the ease with which it dissolves in alcohol. It appears to contain picric acid.

<sup>5</sup> Howland, J., Haessler, F. H., and Marriott, W. McK., *J. Biol. Chem.*, 1916, *xiv*, p. xviii; also personal communication to the author.

ignition.<sup>6</sup> The results indicate that, after drying at 95–100°, the precipitate has the composition  $\text{MgNH}_4\text{PO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$  and that the remainder of the water is driven off when the precipitate is dried at 105°. These assumptions were made in calculating the figures given in the tables.

Because of the small amount of material available, the total "acid-soluble" phosphorus in some of the specimens was determined by the colorimetric method described by the author.<sup>7</sup> In one case the inorganic phosphate was determined by titration of the reprecipitated ammonium phosphomolybdate.

The results obtained are summarized in Table I and clearly indicate that practically all the phosphorus extracted from serum

TABLE I.

*Serum Phosphorus, as Determined by Different Methods, in Mg. per 100 Cc. of Serum.*

Serum from	Total acid-soluble phosphorus			Phosphorus precipitated by Mg mixture in acid extract.			Phosphorus precipitated by molybdate solution			Phosphorus precipitated by Mg mixture in serum.		
	100°.	105°.	Ig-nited	100°.	105°.	Ig-nited	100°.	105°.	Ig-nited	100°.	105°.	Ig-nited.
Sheep.....	11 00	11 13	10.91	10 89	10 53	10 48	10 31	10 30	10 32	10 37	9 89	9 84
" .....	12 01	11 57	12 04	11.40	10 88	10.98						11.88
" .....	8 58		8 60		8 13	8 04	7 66		7 43	7 56		7 58
Beef.....		6 79	7 04				7 01	7 25	6 97	6 31	6 34	6 35
Pig.....	9.70	9.53	9 55			9 09	9 58	9 35	9 38	9 09	9 30	9 02
Dog.....		5 51*			5 62	5 58						
Human.												
M. B. ....		4 74*								4 31	4 17	4 18
I. G. ....		2 61*						2 94†				
Chest fluids....		5 05	4 74								3 56	3 48
		3 83	3 64					2 92	3 01		3 28	3 18
Abdominal fluid...		2 59	2.25					2 05	2 28		2 45	2.29

\* Determined colorimetrically.

† Determined by titration of the reprecipitated phosphomolybdate.

<sup>6</sup> The paper by Jones (*J. Biol. Chem.*, 1916, xxv, 87), in which the results obtained by weighing the air-dried precipitate,  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ , are described, appeared after this work had been completed and the results prepared for publication.

<sup>7</sup> Greenwald, *J. Biol. Chem.*, 1915, xxi, 29.

by dilute acid was in the form of inorganic phosphate or else an extremely labile organic compound. If the latter, it was so unstable that it is extremely difficult to obtain evidence of its existence.

There was, however, a slight difference between the amount of total acid-soluble phosphorus and that precipitated by magnesia mixture or by molybdate solution. The following experiments were performed to ascertain whether or not this difference might be due to the presence of substances that interfered with the precipitation of inorganic phosphate.

A measured volume of serum was dialyzed for 4 or 5 days and the volume again measured. It still contained about 0.3 mg. of acid-soluble phosphorus per 100 cc. of serum. The equivalent of each 100 cc. of the original serum was mixed with a solution containing 0.8 gm. NaCl, 50 mg. urea, 0.1 gm. glucose, 10 mg. creatine, and 7.85 mg. phosphorus (as  $K_2HPO_4$ ). This liquid was then treated in the manner already described. From the results summarized in Table II it is evident that the discrepancies obtained in the first series of experiments cannot be explained on the basis of interference with precipitation by substances in this semi-artificial serum. It is, of course, possible that serum contains some other substance which interferes with the quantitative precipitation of inorganic phosphate. This is not likely and it would seem that phosphorus may occur in a form other than inorganic phosphate and phospholipins. However, the amount of such phosphorus is very small and does not exceed 1 mg. per 100 cc. of serum.

TABLE II.

*Recovery of Phosphate Added to Dialyzed Serum, in Mg. per 100 Cc. of Serum.*

Serum from	Acid-soluble phosphorus in dialyzed serum.	Phosphate added as phosphorus..			Phosphorus precipitated by molybdate solution.			Phosphorus precipitated by Mg mixture in serum.	
		100°.	105°.	Ig-nited.	100°.	105°.	Ig-nited.	105°.	Ig-nited.
Pig.....	0.30*	7.84	7.75	7.85					7.87
Sheep.....	0.28*	7.84	7.75	7.85	7.50	7.50	7.53	7.52	7.62

\* Determined colorimetrically.

## SUMMARY.

The phosphorus compounds of serum consist almost exclusively of phospholipins and inorganic phosphate. There is some indication of the presence of a form which is soluble in dilute acids but is not precipitated by magnesia mixture or by molybdate solution, and which does not dialyze readily from serum.



## THE CHEMICAL NATURE OF THE "VITAMINES."

### I. ANTINEURITIC PROPERTIES OF THE HYDROXYPYRIDINES.\*

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(Received for publication, May 8, 1916.)

The difficulties involved in the isolation of "vitamines" from yeast or rice polishings have proven so serious that success seemed doubtful until a clearer idea should be gained of the chemical nature of these important substances. Accordingly attention has been directed to the preparation and testing of synthetic substances, which appeared likely to display some curative properties similar to the "vitamine fraction" of natural foodstuffs.

Two facts were considered of primary importance in determining the direction of the work. The first of these was that the curative substance seemed to be a pyridine derivative since the curative fraction of rice polishings contains nicotinic acid,<sup>1</sup> and has been found to develop a pyridine-like odor under certain conditions. A second hint was found in the fact that the blue color reaction given by antineuritic foodstuffs when treated with phosphotungstic acid and alkali seems to be rather closely associated with the potent constituents.<sup>2</sup> Since this reaction and the similar reaction with phosphomolybdic acid<sup>3</sup> are known to be

\* A considerable portion of the work here recorded was reported in a paper presented at the Second Pan-American Scientific Congress at Washington, D. C., Jan. 3, 1916, the Proceedings of which have not yet been published.

<sup>1</sup> Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89. Drummond, J. C., and Funk, C., *Biochem. J.*, 1914, viii, 598, and elsewhere.

<sup>2</sup> Drummond and Funk, *Biochem. J.*, 1914, viii, 598. Folin, O., and Macallum, A. B., Jr., *J. Biol. Chem.*, 1912, xi, 265; 1912-13, xiii, 363.

<sup>3</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239. Funk, C., and Macallum, A. B., Jr., *Biochem. J.*, 1913, vii, 356.



produced by substances containing hydroxyl groups in the benzene ring, it was not surprising to find that hydroxyl derivatives of pyridine also give one or both reactions according to the number of the substituted hydroxyl groups.

Accordingly a series of pyridine derivatives was prepared and the therapeutic action of each individual roughly tested on polyneuritic pigeons by the administration of doses of 1 to 10 mg. by intramuscular injection. In some cases doses of 10 to 100 mg. were administered by mouth as a supplementary test. The series included nicotinic, cinchomeric, quinolinic, 6-hydroxynicotinic and citrazinic acids,  $\alpha$ -hydroxypyridine, glutazine, 2,4,6-trihydroxypyridine and its anhydride, and finally 2,3,4-trihydroxypyridine and the so called tetrahydroxypyridine.

Nicotinic, cinchomeric, and quinolinic acids, were prepared by the oxidation of nicotine, quinine, and quinoline respectively;<sup>4</sup> and citrazinic acid from citric acid.<sup>5</sup> The methods of Pechmann and Stokes<sup>6</sup> were followed for the preparation of ethylamino- $\beta$ -hydroxyglutamate, glutazine, 2,4,6-trihydroxypyridine and its anhydride. Tetrahydroxypyridine and 2,3,4-trihydroxypyridine were produced by the method of Ost<sup>7</sup> from the nitroso derivative. In the distillation of meconic acid for this purpose the modification of Peratoner and Leone<sup>8</sup> was used. Better methods than those of Ost are offered by Peratoner and Castellana<sup>9</sup> for the production of hydroxycomenic acid from which by suitable modification of Ost's second method, fair yields of 2,3,4-trihydroxypyridine may be obtained. These methods are being studied as are those of Collie,<sup>10</sup> Tickle and Collie,<sup>11</sup> Lapworth and Collie,<sup>12</sup> and Baron,

<sup>4</sup> Weidel, J., *Ann. Chem.*, 1873, clxv, 330. Weidel, H., and v. Schmidt, M., *Ber. chem. Ges.*, 1879, xii, 1146. Hoogewerff, S., and Van Dorp, W. A., *Rec. trav. chim. Pays-Bas*, 1882, i, 107.

<sup>5</sup> Behrmann, A., and Hofmann, A. W., *Ber. chem. Ges.*, 1884, xvii, 2687.

<sup>6</sup> v. Pechmann, H., and Stokes, H. N., *Ber. chem. Ges.*, 1885, xviii, 2291. Stokes, H. N., and v. Pechmann, H., *ibid.*, 1886, xix, 2694.

<sup>7</sup> Ost, H., *J. prakt. Chem.*, 1879, xix, 203; 1883, xxvii, 257.

<sup>8</sup> Peratoner, A., and Leone, R., *Gazz. chim. ital.*, 1894, xxiv, pt. ii, 75.

<sup>9</sup> Peratoner, A., and Castellana, V., *Gazz. chim. ital.*, 1906, xxxvi, pt. i, 21.

<sup>10</sup> Collie, J. N., *J. Chem. Soc.*, 1891, lix, 617.

<sup>11</sup> Tickle, T., and Collie, J. N., *J. Chem. Soc.*, 1902, lxxxii, 1004.

<sup>12</sup> Lapworth, A., and Collie, J. N., *J. Chem. Soc.*, 1897, lxxi, 838.

Remfry, and Thorpe.<sup>13</sup> Coumalic acid was used for making hydroxynicotinic acid,<sup>14</sup> by the distillation of which  $\alpha$ -hydroxypyridine is readily obtained.<sup>15</sup>

The test animals used were pigeons, in which polyneuritis was developed by feeding *ad libitum* on white rice. In all cases the disease ran its course till the animals were unable to stand or make controlled movements of the legs or wings. In such cases death usually follows within 24 hours and may occur at any moment. A number of times birds died in the experimenter's hands before treatment could be administered. During and after treatment the birds were continued on a diet of white rice, and under such conditions a redevelopment of the symptoms can be only a matter of a few days. Since forced feeding was not resorted to on account of the time necessary to care for a large number of birds in this way, it was occasionally the case that the pigeons became weak and emaciated before the characteristic symptoms developed to the desired point. Such cases are not of course strictly comparable with those of sudden development in which the animals retain a great deal of their original strength and vigor, though losing muscular control, and a measure of uncertainty is thereby introduced in occasional cases.

On treating polyneuritic pigeons with the substances above mentioned definite evidence of curative power was noted in the case of  $\alpha$ -hydroxy-, 2,4,6-trihydroxy-, and 2,3,4-trihydroxypyridine. The remainder of the series showed no effect. But as will be seen later, such negative evidence in the case of any hydroxypyridine derivative cannot be regarded as conclusive. The first of the curative substances tested was  $\alpha$ -hydroxypyridine. Three birds were treated with excellent results. However, three others treated later showed little or no improvement. On proceeding with the series to the polyhydroxy compounds, a rapid striking cure was obtained with a preparation of 2,4,6-trihydroxypyridine, followed by several partial or complete failures. A second and third fresh preparation, however, produced two and three fairly rapid cures respectively. A single fresh preparation

<sup>13</sup> Baron, H., Remfry, F. G. P., and Thorpe, J. F., *J. Chem. Soc.*, 1904, lxxxv, 1726.

<sup>14</sup> v. Pechmann, H., and Welsh, W., *Ber. chem. Ges.*, 1884, xvii, 2384.

<sup>15</sup> v. Pechmann, H., and Baltzer, O., *Ber. chem. Ges.*, 1891, xxiv, 3144.

of 2,3,4-trihydroxypyridine was administered to three pigeons simultaneously at the close of the working day, one pigeon receiving 2, the second 1, and the third 0.5 mg. The following morning the first bird was dead, while the second and third had completely recovered from all paralytic symptoms. Subsequently the same material failed to benefit several birds to which it was administered.

In each case all the cures obtained were of those pigeons which were first treated with a given preparation, while those treated with the same preparation a few days or weeks later invariably received no benefit. It was obvious that the substances had changed in some manner so as to lose the curative power. As there was no evidence of decomposition, it seemed probable that it was due to isomerisation. Such rearrangements have greatly interested organic chemists for many years, but no definite evidence has been adduced that such transformations take place in the tissues as a fundamental part of a biological process, though they appear to be just such reactions as might easily occur within the living organism. Many of them take place with the greatest ease and without resort to the vigorous methods ordinarily employed in our laboratories.

The existence of a tautomerism in the hydroxypyridines has long been recognized in a general way. It has been observed that these substances under certain circumstances react as hydroxy or enol compounds, under others as ketones. However, no one has regarded it possible that two isomers could exist in a free isolated state as two distinct individuals. Each of the hydroxypyridines is described in the literature as one substance of definite though dual properties. A critical examination of the literature, however, suggested that this was not the case. It was therefore decided to study the isomerism of some one of the curative substances more thoroughly than had been done previously. For this purpose  $\alpha$ -hydroxypyridine was chosen. This substance has been administered to about eighty pigeons under varying conditions and certain important facts have come to light.

It is prepared by fusing 6-hydroxynicotinic acid and distilling the residue after the evolution of carbon dioxide has ceased. The oily distillate presently solidifies to a mass made up of either granular or needle crystals, or both, depending apparently on the

rate of cooling and the presence or absence of the merest traces of moisture. The residue condensing in the neck of the distilling flask after completion of the process always crystallizes in the form of needles. A crystalline granular mass so obtained on being melted and kept at a temperature well above the melting point for some time and allowed to cool slowly, again solidifies to a mass, now consisting largely of needles. If this remelted mass is dissolved in benzene and recrystallized therefrom by the addition of ligroin, needles are found to predominate in the beautiful crystalline precipitate. Sometimes the needles may be obtained absolutely free from granules. From the mother liquor on concentration, granular crystals separate and may be obtained in a pure form by filtering from the hot solvent, as the small residuum of needles dissolves freely in the hot mixture of ligroin and benzene. Both forms after washing freely with petroleum ether and drying rapidly in a vacuum desiccator, melt sharply at 106–107°. A fused mixture does not melt sharply and the melting point varies with the speed with which the temperature is raised. Evidently granular crystals are converted partially into needles by dry heat. On allowing the solid needle form to stand for a few days in an open vessel or cork-stoppered bottle, it will be found that the crystal form has undergone a change. The needles which were originally clear and sharply defined are marked by transverse lines of cleavage. In the course of 10 to 20 days they become roughly needle-shaped granular aggregates. The rate of this change is variable but seems to depend greatly on the amount of moisture in the atmosphere surrounding the crystals. By exclusion of moisture the transition takes place much more slowly. A water solution of needles on evaporation deposits only granules which, however, may be partially reconverted into needles by dry heat.

If the two forms of crystals are titrated in the cold with alcoholic bromine according to the method of Kurt Meyer,<sup>16</sup> neither absorbs appreciable amounts of bromine instantaneously. The granular form absorbs bromine gradually and the needle form still more slowly. Neither form can therefore be the hydroxy or enol form. However, on dissolving either form in an excess of

<sup>16</sup> Meyer, K. H., *Ann. Chem.*, 1911, cccxxx, 212.

alcoholic sodium hydroxide of known strength, and titrating in the cold with bromine, instantaneous absorption occurs in excess of that required by the caustic soda. In the presence of from five to ten molecular equivalents of sodium hydroxide, from 50 to 60 per cent of the theoretical quantity of bromine is absorbed with great rapidity.  $\alpha$ -Hydroxypyridine in alcoholic sodium hydroxide solution is unquestionably largely, though not wholly, in the enol form. Presumably all the metallic salts are likewise enols, a presumption which is in accord with the known facts in regard to other enol-keto tautomers. If the cold alcoholic solution of the sodium salt is just neutralized with cold alcoholic hydrochloric acid detectable amounts of the free enol form are momentarily present as determined by bromine titration. The existence of any considerable proportions of the enol form in the free state appears to be brief in all neutral solvents and no method has been found for its isolation. Since  $\alpha$ -hydroxypyridine is a base as well as an acid, it forms well defined salts with strong acids. Whether these salts are partly salts of the enol form cannot be determined by titrimetric methods, since the presence of the acid makes the determination of an end-point impossible. If these salts are not partly enols they are at least transformed into enols with the greatest rapidity during titration.

It appears that there exist not two but three isomeric forms of  $\alpha$ -hydroxypyridine for which we may readily imagine structural formulas<sup>17</sup> expressing intramolecular rearrangement. This matter will be taken up in a later paper. By simple means we may cause any desired form to predominate and may pass through the cycle repeatedly. Each exists in equilibrium with at least one other in relative quantities depending on conditions. We shall understand their relationships fully only after finding a method for determining each of the three forms quantitatively under varying conditions. At present we can measure only the amount of enol.

To a degree it has been possible to determine the curative power of each of the three forms. Several preparations of freshly crystallized needles dissolved in water immediately before injection have been administered to fourteen polynuritic pigeons in

<sup>17</sup> Kauffmann, H., *Ber. chem. Ges.*, 1903, xxxvi, 1062. Decker, H., *J. prakt. Chem.*, 1900, lxii, 266.

doses of 1 to 2 mg. Improvement or cure resulted in every case as evidenced by change in weight, paralytic symptoms, and appetite. Eight of the birds reached an optimum from 1 to 3 days after treatment and after a single dose of 1 to 2 mg. lived from 5 to 10 days. Three birds were partially cured a second and third time after redevelopment of severe symptoms and lived 11, 13, and 15 days respectively after the first onset of the disease. However, six cures differed markedly from the others obtained with  $\alpha$ -hydroxypyridine. The first evidences of improvement were noticed in these cases in 40 minutes to 2 hours after injection, when the animals began to assume nearly normal attitudes. At intervals brief spasms of spastic movement occurred, which gradually became less frequent and less prolonged and in the course of 3 to 12 hours ceased altogether. After this time the birds did not differ markedly in condition from those in which less rapid cures were obtained. The gain in weight and other evidences of improvement were of the same character. The rapid cures obtained with 2,4,6-trihydroxypyridine above reported were attended by the same symptoms. In general it may be said that the results obtained with these substances approximate very closely those obtained with hydrolyzed extract of rice polishings, in both rapidity and completeness of action on the paralytic symptoms. The ultimate effects on the general health and weight of the birds were less satisfactory than those of the natural "vitamines." Protective experiments on healthy birds on a white rice diet have not yet been made. Water solutions of the needle form kept at ordinary temperatures retain some curative power for 5 or 6 days, after which it is no longer detectable. At boiling temperature the curative power disappears very rapidly.

The physiological effect of the enol form can only be guessed, as long as it remains impossible to obtain it free from the other forms and from extraneous substances. Injections of 1 mg. in caustic soda solution produced fairly rapid cures in two birds so treated. Administration by mouth of 10 mg. in caustic soda followed by dilute acetic acid also effected fairly rapid cures. The silver salt was also administered to three pigeons in doses of 20 mg. each followed by salt solution as an antidote for the silver. Two had recovered from the paralysis the following day and lived 3 and 7 days respectively. One died 5 hours after treatment.

No very satisfactory conclusion can be drawn from these experiments on account of the disturbing factor of the toxicity of caustic soda and silver. The former produced severe inflammation of the tissues with which it came in contact and the latter evidenced a general toxic action, as was to be expected. Nevertheless there was indubitable relief of the typical paralytic symptoms. This fact that solutions of  $\alpha$ -hydroxypyridine in caustic soda are curative led to much confusion, as it suggested that the curative form was the enol. However, since hydroxypyridine under such conditions is only partly enol, as indicated by bromine titration, the curative property of caustic soda solution may be due to the non-enolic portion.

The granular form as obtained by prolonged standing of the needle form has been tested on some thirty birds under varying conditions. Doses of from 0.5 to 100 mg. have been tried by injection and by mouth. Injections in water have been made immediately after solution, after standing several days, after boiling, and after heating under steam pressure. Injections in mineral oil and in fatty acid have also been tried. In no case was there the slightest evidence of any benefit. When large doses were administered, the action seemed distinctly adverse in several cases, as the birds died sooner than was expected. Doses of 10 mg. injected into healthy birds, however, produced no gross toxic symptoms.

This absolute lack of curative properties is striking and suggestive. If the isomeric forms exist in equilibrium and there is fairly rapid transformation in any direction, one would suppose that the granular form would, when injected into the body, rapidly convert itself into the curative form as the latter was removed from solution by absorption in the tissues or fluids requiring it. That such is not the case suggests strongly that the pathological conditions of polyneuritis are not due to a deficiency of a substance *per se*, but to a lack of a certain type of potential energy which only certain substances can supply. In other words, one is led to surmise that it is the potentiality of isomeric change that produces the desired result. In this connection it is interesting to note that many purine and pyrimidine derivatives, some of which Funk has reported to be partially curative for poly-

neuritis,<sup>18</sup> are also theoretically capable of a similar isomerism. That these substances are to a greater or less degree enol-keto tautomers is strongly indicated, by well known reactions of uric acid for example. The existence of a third isomer is not excluded by theoretical considerations, but lacks the support of any considerable experimental evidence.

It appeared not unlikely that other hydroxypyridines might prove more easily controlled. Accordingly  $\beta$ - and  $\gamma$ -hydroxypyridine and  $\gamma$ -lutidone have been prepared and are being studied. They show curative properties only under certain conditions, and these curative properties automatically disappear with the lapse of time. Each crystallizes in two different forms and forms metallic salts which absorb bromine rapidly in cold alcohol. Present evidence indicates that they are strict analogues of  $\alpha$ -hydroxypyridine.

The antineuritic properties of these substances suggest that an isomerism is at least partially responsible for the instability of the "vitamines" in foodstuffs, and that the antineuritic property may be inherent in the potentiality of this type of isomerism. We may not conclude that "vitamines" are necessarily hydroxypyridines since a similar isomerism may exist in substances containing other heterocyclic nitrogenous nuclei which are known to occur widely as constituents of animal tissue.

<sup>18</sup> Funk, C., *J. Physiol.*, 1912-13, xlv, 489.





# STUDIES IN CARBOHYDRATE METABOLISM.

## XI. THE RÔLE OF CALCIUM IN THE REGULATION OF BLOOD SUGAR CONTENT.

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A perusal of the literature gives one the impression that calcium salts may play a more or less important rôle in the regulation of carbohydrate metabolism even though their possible function is recognized only in a vague, indefinite manner. On the one hand, Bunge<sup>1</sup> has put forth a warning against a sugar-rich diet deficient in salts, especially iron and calcium, the inference being that such a deficiency may have an influence on the production of diabetes. This is especially pertinent in view of the fact that diabetes is said<sup>2</sup> to be associated with an increased output of calcium. Again, it is well known that the glycosuria evoked by long continued injection of sodium chloride into the blood may be checked promptly by the intravenous administration of dilute solutions of calcium chloride.<sup>3</sup> On the other hand, the introduction into the blood of strong<sup>4</sup> solutions of calcium chloride is sufficient to induce the appearance of sugar in the urine.

Schrank<sup>5</sup> has claimed that calcium salts will either prevent or inhibit the glycosuria called forth by epinephrin.

Calcium salts have also been associated closely with the function of the thyroid-parathyroid mechanism, MacCallum and

<sup>1</sup> v. Bunge, G., *Z. Biol.*, 1901, xli, 155.

<sup>2</sup> Compare Allen, F. M., *Studies concerning Glycosuria and Diabetes*, Boston, 1913.

<sup>3</sup> For literature see Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905-06, xv, 321.

<sup>4</sup> Underhill, F. P., and Kleiner, I. S., *J. Biol. Chem.*, 1908, iv, 395.

<sup>5</sup> Schrank, F., *Z. klin. Med.*, 1909, lxvii, 230.

Voegtlin<sup>6</sup> having demonstrated a loss of calcium to the body after extirpation of this apparatus together with cessation of tetany symptoms subsequent to calcium injections. Underhill and Blatherwick<sup>7</sup> showed that during the tetany attendant upon thyreoparathyroidectomy the blood sugar content is markedly diminished, and in a later paper<sup>8</sup> evidence was presented, confirming the results of MacCallum and Voegtlin, that calcium checks tetany, and furthermore, demonstrating that the blood sugar content likewise may be restored to normal temporarily by calcium administration. It was stated:

"It is apparent from these results that in tetany calcium plays an important rôle in maintaining the equilibrium of the sugar-regulating mechanism, for when there is a sufficiency of calcium blood sugar content is normal. Conversely, blood sugar content tends to become low when there is an apparent deficiency of calcium."<sup>9</sup>

The data of the present communication were obtained in an endeavor to determine the influence which calcium salts may exert upon the blood sugar content, first, under normal conditions, and, second, under circumstances in which the equilibrium of the blood sugar-regulating mechanism had been disturbed experimentally. For the latter purpose epinephrin was employed.

### Methods.

Normal full grown rabbits maintained under practically uniform conditions served as experimental animals. Only a single experiment was performed upon each animal. This condition was enforced because of the well known fact that epinephrin may fail to elicit the maximum effect subsequent to the initial injection. The epinephrin employed was the adrenalin of Parke, Davis and Company, 1 : 1,000. Maximum doses, 1 cc. (1 mg.) per kilo of body weight, were administered. Blood sugar was estimated by the Forschbach and Severin<sup>10</sup> method, which has been found to yield results in good agreement with the procedure of Waymouth Reid.<sup>11</sup> Sugar in the urine was determined gravimetrically by the Allihn method.

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<sup>6</sup> MacCallum, W. G., and Voegtlin, C., *J. Exp. Med.*, 1909, xi, 18.

<sup>7</sup> Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii,

<sup>8</sup> Underhill and Blatherwick, *J. Biol. Chem.*, 1914, xix, 119.

<sup>9</sup> Underhill and Blatherwick, *J. Biol. Chem.*, 1914, xix, 125.

<sup>10</sup> Forschbach and Severin, *Arch. exp. Path. u. Pharm.*, 1912, lxxviii, 341.

<sup>11</sup> Reid, E. W., *J. Physiol.*, 1896, xx, 316.

*The Influence of Subcutaneous Injections of Calcium Lactate and Calcium Chloride upon Blood Sugar Content.*

From the data in Table I it may be seen that the subcutaneous administration of small or large doses of calcium salts (lactate and chloride) is without significant influence upon the blood sugar content under the experimental conditions recorded.

TABLE I.

*The Sugar Content of the Blood after Subcutaneous Injections of Calcium Lactate and Calcium Chloride.*

Rabbit.	Body weight.	Blood sugar content (in percentages)							Remarks.
		Nor-mal.	Hours after calcium injection						
			1	2	3	4	5	6	
	gm.								
22	2,200	0 16	0 17	0 17	0 13	0 11	0 15	0 16	Injection of 10 cc. 3 per cent calcium lactate.
23	2,200	0 12	0 15	0 17	0 16	0 10	0 15	0 15	
26	2,000	0 12	0 10	0 12	0 13	0 15	0 12		Injection of 20 cc. 3 per cent calcium lactate.
27	2,300	0 11	0 12	0 15	0 12	0 12	0 12		
76	2,400	0 14	0 15	0 14	0 17	0 12	0 12	0 11	Injection of 25 cc. 11 per cent calcium chloride.
77	2,400	0 15	0 14	0 11	0 14	0 11	0 11	0 13	
Average...		0 13	0 14	0 14	0 14	0 12	0 13	0 14	No reducing substance in any urine.



CHART 1. Curve showing the sugar content of the blood after injections of calcium lactate and calcium chloride. Average of four experiments (Table I).

*The Sugar Content of the Blood and Urine under the Influence of Epinephrin.*

It is a well known fact that the same dose of epinephrin may not produce the same degree of glycosuria in two different animals or in the same animal at different periods. Therefore it is decidedly difficult to establish a standard which may be accepted as an average amount of sugar appearing in the urine under the stimulus of a given dose of epinephrin. With these limitations in mind four animals were taken at random from stock, all were given the same quantity of epinephrin per kilo, and the average amount of sugar excreted by the kidneys was accepted as the standard for epinephrin glycosuria. In like manner blood sugar estimations carried through at hourly periods were averaged for the same four animals. From the figures obtained a curve, Chart 2, has been plotted which represents the blood sugar changes subsequent to epinephrin treatment. As may be seen from Table II, the extent of glycosuria and the degree of hyperglycemia are distinctly variable, but the average figures are undoubtedly a fair representation of epinephrin glycosuria and hyperglycemia. A glance at the chart will show clearly that the hyperglycemia attains its maximum between the 3rd and 5th hours, gradually decreasing and approaching normal again at about the 8th hour. Approximately 1.5 gm. of sugar represent the average output under the influence of 1 mg. of epinephrin per kilo of body weight.

TABLE II.

*The Sugar Content of the Blood and Urine under the Influence of Epinephrin.*

Epinephrin.									
Rabbit.	Body weight.	Blood sugar content (in percentages).							Sugar in urine <sup>a</sup>
		Normal.	Hours after epinephrin injection.						
			1	2	3	5	6	7½	
	gm.								gm
3	2,100	0.13	0.27	0.40	0.50	0.27	0.30	0.21	0.46
4	2,000	0.10	0.50	0.49	0.42	0.40	0.36	0.28	1.58
5	2,000	0.12	0.30	0.39	0.53	0.40	0.26	0.12	1.55
6	2,000	0.18	0.36	0.43	0.41	0.43	0.36	0.16	2.34
Average.....		0.13	0.36	0.43	0.46	0.37	0.32	0.19	1.49

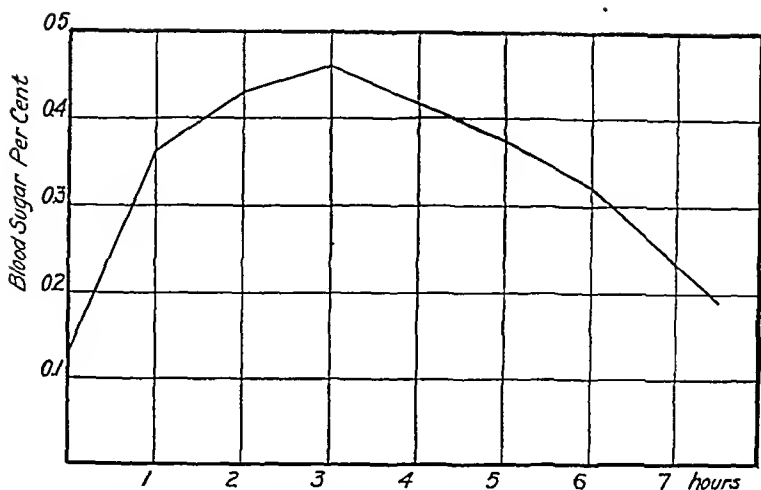


CHART 2. Curve showing the sugar content of the blood after injections of epinephrin. Average of four experiments (Table II).

*The Influence of Various Doses of Calcium Salts upon the Hyperglycemia and Glycosuria Provoked by Epinephrin.*

As far as I am aware the only investigation of the influence of calcium salts upon epinephrin glycosuria is that reported by Schrank.<sup>5</sup> He made determinations of the sugar in the urine only and claimed that calcium chloride markedly diminishes, or may entirely inhibit, epinephrin glycosuria. In his experiments the animals were interchanged in some instances; that is, those rabbits receiving epinephrin alone were employed later for experiments in which both epinephrin and calcium were given, and conversely. Relatively small quantities of epinephrin were administered, 0.8 to 1.0 cc. per animal, body weight varying from 850 to 2,720 gm.

Throughout this investigation the calcium salts were injected subcutaneously at varying periods previous to epinephrin administration in order to obtain the maximum influence of the calcium salt which is presumably rather difficult of absorption. The influence which calcium exerts upon epinephrin glycosuria and hyperglycemia is to be seen in Table III and Charts 3, 4, 5, 6, and 7, given below. In the first series of experiments, Table III,

TABLE III.

*The Influence of Various Doses of Calcium Salts upon the Hyperglycemia and Glycosuria Provoked by Epinephrin.*

Rabbit.	Body weight.	Blood sugar content (in percentages).								Sugar in urine.
		Normal.	Hours after epinephrin injection.							
			1	2	3	4	5	6	7½	
Subcutaneous injection of 10 cc. 1 per cent calcium chloride ½ hour previous to epinephrin.										
	gm.									gm.
8	2,000	0.13	0.32	0.37	0.30		0.29	0.19	0.11	3.03
9	2,300	0.16	0.33	0.41	0.39		0.33	0.27	0.22	2.05
10	2,200	0.11	0.34	0.44	0.54		0.43	0.29	0.14	3.28
Average..		0.13	0.33	0.41	0.41		0.35	0.25	0.16	2.80
Subcutaneous injection of 10 cc. 1 per cent calcium lactate 2 hours previous to epinephrin.										
13	1,800	0.15	0.54	0.52	0.37		0.30	0.19	0.15	2.83
14	2,200	0.15	0.54	0.53	0.39		0.25	0.18	0.14	5.20
Average..		0.15	0.54	0.52	0.38		0.28	0.19	0.15	4.01
Subcutaneous injection of 10 cc. 3 per cent calcium lactate 2 hours previous to epinephrin.										
15	2,000	0.10	0.47	0.46	0.28		0.27	0.23	0.15	4.57
16	1,800	0.15	0.40	0.49	0.29		0.29	0.25	0.18	3.60
24	2,200	0.18	0.51	0.44	0.31	0.28	0.25	0.24	0.19	7.60
25	2,000	0.15	0.46	0.44	0.31	0.30	0.28	0.24	0.12	5.82
Average..		0.15	0.46	0.46	0.30	0.29	0.27	0.24	0.16	5.39
Subcutaneous injection of 10 cc. 3 per cent calcium lactate 3 hours previous to epinephrin.										
20	2,500	0.17	0.47	0.43	0.53	0.36	0.30	0.19		5.70
21	2,000	0.16	0.44	0.40	0.57	0.37	0.26	0.18		2.87
Average..		0.17	0.46	0.42	0.55	0.37	0.28	0.19		4.28
Subcutaneous injection of 10 cc. 3 per cent calcium lactate 4 hours previous to epinephrin.										
18	2,200	0.12	0.33	0.31	0.25	0.25	0.18			2.67
19	2,200	0.15	0.25	0.39	0.40	0.27	0.17			3.01
Average..		0.14	0.29	0.35	0.33	0.26	0.18			2.85

Rabbits 8, 9, and 10, Chart 3, the effect of a 1.0 per cent solution of calcium chloride given  $\frac{1}{2}$  hour previous to epinephrin may be observed. The amount of sugar appearing in the urine has been markedly increased. Chart 3 shows that calcium chloride injected under the cited conditions has left unchanged the general character of the epinephrin hyperglycemia curve.

When the same dose of calcium lactate was introduced 2 hours previous to epinephrin, glycosuria was even more marked (Table III, Rabbits 13 and 14) and the curve of hyperglycemia was

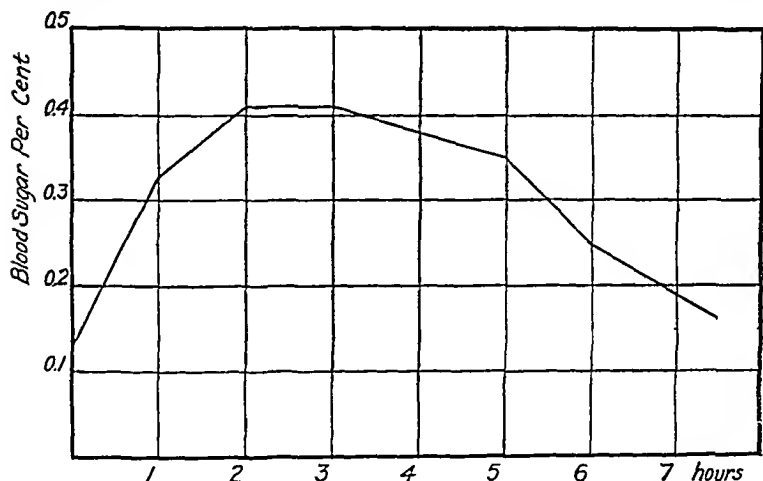


CHART 3. Curve showing the influence of the subcutaneous injection of 10 cc. of 1.0 per cent calcium chloride solution upon epinephrin hyperglycemia. Calcium was injected  $\frac{1}{2}$  hour previous to epinephrin. Average of three experiments (Table III, Rabbits 8, 9, and 10).

radically changed (Chart 4), the maximum being attained sooner; that is, between the 1st and 2nd hours instead of between the 3rd and 5th hours. Injection of a larger quantity of calcium lactate 2 hours previous to epinephrin administration caused a still greater average output of sugar (Table III, Rabbits 15, 16, 24, and 25), the general character of the hyperglycemia curve (Chart 5) remaining comparable to that of Chart 4. Apparently the injection of 10 cc. of 3.0 per cent calcium lactate 2 hours previous to epinephrin treatment represents the optimum conditions under



the limitations of this investigation, for after the subcutaneous introduction of 10 cc. of a 3.0 per cent solution of calcium lactate 3 hours previous to epinephrin injection the hyperglycemia curve (Chart 6) shows a tendency to return to that of the standard epinephrin curve, the maximum elevation occurring between the 3rd and 4th hours. Likewise the average quantity of sugar excreted is diminished (Table III, Rabbits 20 and 21). This

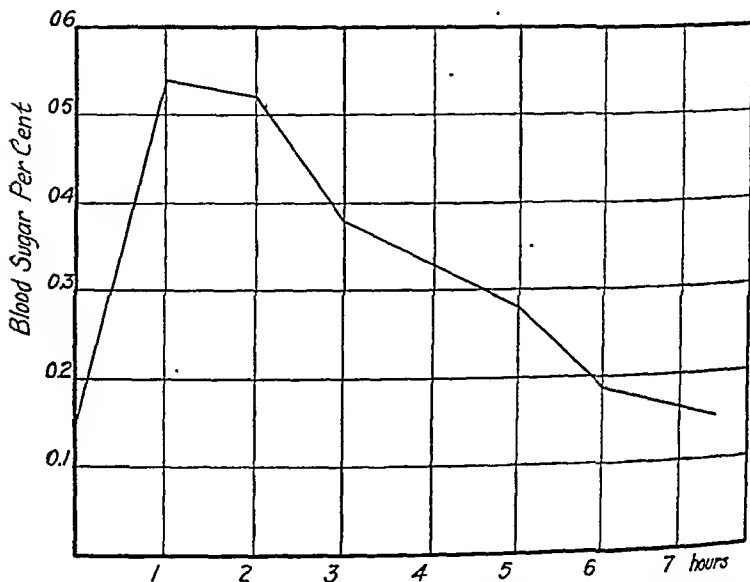


CHART 4. Curve showing the influence of the subcutaneous injection of 10 cc. of 1.0 per cent calcium lactate solution upon epinephrin hyperglycemia. Calcium was injected 2 hours previous to epinephrin. Average of two experiments (Table III, Rabbits 13 and 14).

return to the standard epinephrin curve is still further emphasized by an inspection of Chart 7 which shows the influence of 10 cc. of a 3.0 per cent solution of calcium lactate injected 4 hours previous to epinephrin treatment. The average sugar output is also still further diminished (Table III, Rabbits 18 and 19).

These results make it apparent that calcium salts, although devoid of any measurable influence upon blood sugar content

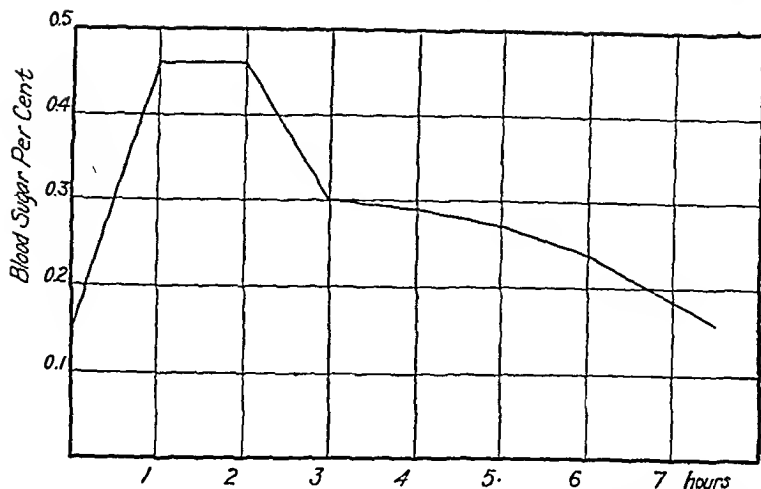


CHART 5. Curve showing the influence of the subcutaneous injection of 10 cc. of 3.0 per cent calcium lactate solution upon epinephrin hyperglycemia. Calcium was injected 2 hours previous to epinephrin. Average of four experiments (Table III, Rabbits 15, 16, 24, and 25).

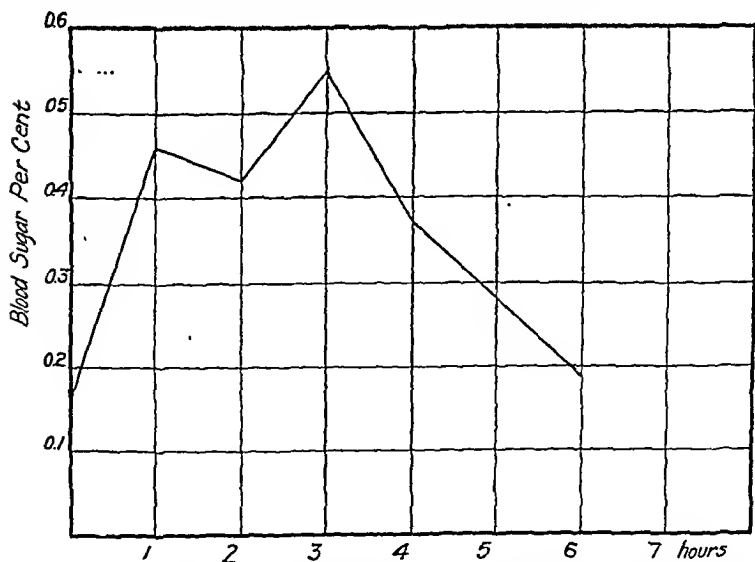


CHART 6. Curve showing the influence of the subcutaneous injection of 10 cc. of 3.0 per cent calcium lactate solution upon epinephrin hyperglycemia. Calcium was injected 3 hours previous to epinephrin. Average of two experiments (Table III, Rabbits 20 and 21).

when introduced into normal rabbits, may show an appreciable effect when administered suitably to animals with a subsequently disturbed equilibrium of the sugar-regulating mechanism. It may therefore be concluded that the subcutaneous injection of calcium lactate may distinctly change the character of the curve of epinephrin hyperglycemia and markedly increase the amount of sugar appearing in the urine under the stimulus of this glycosuria-producing drug.

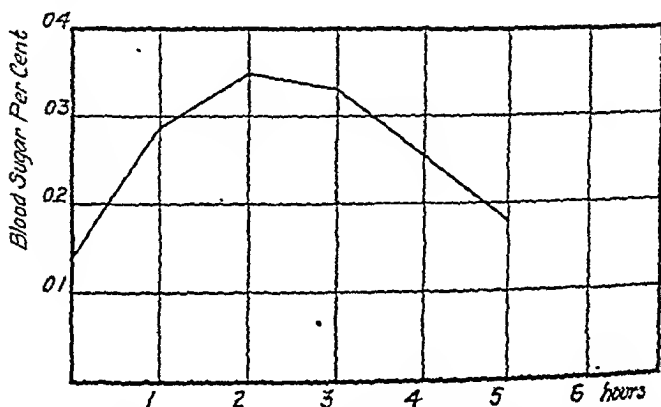


CHART 7. Curve showing the influence of the subcutaneous injection of 10 cc. of 3.0 per cent calcium lactate solution upon epinephrin hyperglycemia. Calcium was injected  $\frac{1}{4}$  hours previous to epinephrin. Average of two experiments (Table III, Rabbits 18 and 19).

*The Influence of Sodium Phosphate and of Sodium Oxalate upon Blood Sugar Content and upon Epinephrin Hyperglycemia and Glycosuria.*

In a recent paper Starkenstein<sup>12</sup> has claimed that the introduction of orthophosphoric, metaphosphoric, pyrophosphoric, phytic (inosite-phosphoric), and citric acids when administered as sodium salts to animals, by mouth, subcutaneously, or intravenously, calls forth symptoms which include muscle twitching, tremor, and convulsions. This influence is ascribed to their common function as calcium precipitants. The symptoms evoked in this manner may be relieved by calcium administration.

<sup>12</sup> Starkenstein, E., *Arch. exp. Path. u. Pharm.*, 1914, lxxvii, 45.

Greenwald<sup>13</sup> has repeated some of these experiments and has reported his failure to obtain the effects mentioned by Starkenstein.<sup>14</sup>

Leaving out of consideration the question whether these sodium salts cause tetany symptoms, it may be accepted that their introduction into the organism will undoubtedly act to rob it of calcium. If calcium injections will increase epinephrin glycosuria and will alter the character of the curve of epinephrin hyperglycemia, what effect upon blood sugar content may be expected when calcium is withdrawn from the normal organism? Or, if there is no appreciable change in blood sugar content, will the withdrawal of calcium change the features of epinephrin hyperglycemia and glycosuria? The results obtained by subcutaneous and intravenous administration of sodium phosphate upon the blood sugar content of normal rabbits may be seen in Table IV. Whichever method of introduction is employed it is evident that blood sugar content may be appreciably lowered temporarily. This influence is not invariable, however, for some animals fail to respond; for example, Rabbit 105. With intravenous injections the effect appears sooner and is more transient than with subcutaneous administration. It is possible, therefore, that withdrawal of calcium from the body will cause a distinct lowering of the sugar in the blood. However, before ascribing this influence to calcium withdrawal a second factor must be considered; namely, the alkalinity of the phosphate solution. To determine whether the diminished blood sugar content is due to the withdrawal of calcium or to the introduction of alkali, experiments were carried through with a neutral calcium-precipi-

<sup>13</sup> Greenwald, I., *J. Pharm. and Exp. Therap.*, 1915, vii, 57.

<sup>14</sup> In my hands the subcutaneous injection of sodium phosphate ( $\text{Na}_3\text{PO}_4$ ) in the doses employed in this investigation never produced any symptoms whatsoever. If intravenous introduction was made too rapidly, respiratory difficulties were in evidence. On the other hand, even though no symptoms were apparent during the period of injection, striking effects were seen about  $\frac{3}{4}$  of an hour later. These consisted of peculiar head movements, the nose being held high in the air. These motions remind one strongly of those sometimes seen in dogs in incipient tetany after thyro-parathyroidectomy; they last for about 1 hour and then gradually cease. But even for an hour or more after the head has been held quiet the movements may be initiated again for a brief period by disturbing the animal, picking it up, etc.

tating salt, sodium oxalate (Table V). In this instance the initial action is definitely to increase the sugar of the blood, which may later, in certain experiments at least, fall below normal. It is

TABLE IV

*The Influence of Subcutaneous and Intravenous Injections of Sodium Phosphate upon Blood Sugar Content.*

Rab bit	Body weight	Blood sugnr content (in percentages)									Remarks
		Nor- mal	Hours after phosphate injection								
			1	2	3	4	5	6	7		
	gm										Subcutaneous injections
38	2,000	0 16	0 29	0 12	0 07	0 06	0 02	0 10	0 11		10 cc 0.5 N Na <sub>2</sub> PO <sub>4</sub>
39	3,000	0 17	0 22	0 09	0 06	0 04	0 10	0 12	0 11		15 " 0.5 " "
41	2,800	0 15	0 15	0 14	0 12	0 02	0 03	0 08	0 10		15 " 0.5 " "
104	2,800	0 16	0 12	0 08	0 09	0 12	0 13				20 " 0.5 " "
105	1,800	0 14	0 15	0 11	0 11	0 14	0 12				10 " 0.5 " "
											Intravenous injections
96	2,400	0 14	0 10	0 04	0 02	0 13	0 14				100 cc 0.1 M Na <sub>2</sub> PO <sub>4</sub> in
97	2,800	0 14	0 16	0 05	0 05	0 14	0 12				4 to 6 minutes in each
100	2,000	0 16	0 16	0 04	0 08	0 10					instance
101	2,000	0 16	0 15	0 08	0 16	0 17					

TABLE V

*The Influence of Subcutaneous Injections of Sodium Oxalate upon Blood Sugar Content*

Sugar Content													
Rabbit	Body weight	Blood sugar content (in percentages)									Sugar in urine	Injection of 0.5% sodium oxalate	
		Normal	Hours after oxalate injection										
			1	2	3	4	5	6	7	8			9
	gm										gm	cc	
28	2,000	0 18	0 23	0 29	0 31		0 17		0 04	0 07	0 06	Present	10
29	2,200	0 18	0 23	0 20	0 15		0 13		0 08	0 03		Absent	20
30	2,700	0 15	0 22	0 25	0 17	0 17	0 18		0 15	0 03	0 03	0 08	10
31	2,300	0 14	0 27	0 30	0 23	0 23	0 23		0 18	0 02	0 04	0 20	10
32	2,200	0 16		0 19		0 16		0 10		0 10		Absent	10
33	2,000	0 16		0 23		0 17		0 11		0 10		"	10
34	2,000	0 17	0 20		0 14		0 11	0 12	0 14	0 15	0 17	"	10
35	2,000	0 17		0 30		0 19		0 17	0 16	0 23	0 27	Present	15
36	2,200	0 15		0 18		0 14		0 03	0 04	0 06		0 07	10
37	2,200	0 17		0 22		0 17		0 17	0 10	0 13	0 17	Absent	10

evident, therefore, that the apparent withdrawal of calcium will cause a fall in blood sugar content. Parenthetically it may be stated that alkali will produce the same result, and a discussion of the possibilities as to the manner in which these facts may be correlated will be discussed in a later paper.

In order to demonstrate even more conclusively the influence that calcium withdrawal may exert upon blood sugar changes, experiments were so devised that sodium phosphate solutions

TABLE VI.

*The Influence of Subcutaneous Injections of Sodium Phosphate upon the Hyperglycemia and Glycosuria Provoked by Epinephrin.*

Rabbit	Body weight	Blood sugar content (in percentages)										Sugar in urine	Injection of 0.5 N sodium phosphate	
		Normal	Hours after injection of											
			Sodium phosphate					Epinephrin						
			1	2	3	4	1	2	3	4	5			
	gm.										gm	cc		
48	2,100	0.16	0.16				0.20	0.34	0.29	0.21	0.15	2.69	10	
49	2,800	0.15	0.23				0.26	0.40	0.31	0.27	0.16	5.24	15	
Average		0.15					0.23	0.37	0.30	0.24	0.15	3.96		
46	2,400	0.11	0.12	0.13			0.38	0.30	0.28	0.19	0.16	0.99	10	
47	2,000	0.14	0.13	0.13			0.28	0.28	0.26	0.21	0.19	5.01	10	
Average		0.13					0.33	0.29	0.27	0.20	0.17	3.00		
43	2,000	0.18	0.15	0.15	0.14	0.05	0.23	0.28	0.33	0.25	0.17	1.64	10	
45	2,000	0.17	0.16	0.16	0.18		0.24	0.30	0.30	0.25	0.18	1.19	10	
50	2,500	0.15	0.13	0.14	0.06	0.04	0.12	0.29	0.22	0.16		0.67	10	
Average		0.16					0.19	0.29	0.28	0.22	0.17	1.16		

were injected at varying periods prior to epinephrin administration. The results are to be found in Table VI. It will be observed that when phosphate is introduced 1 hour previous to epinephrin injection (Experiments 48 and 49) there is a less marked degree of hyperglycemia than in the standard epinephrin curve. Moreover, the normal is regained more rapidly. In spite of these facts the total sugar output was higher than when epinephrin was given alone (compare with Table I). Epinephrin injected 2 hours after administration of sodium phosphate (Experi-

ments 46 and 47) yielded the same type of curve as those of Experiments 48 and 49. - Here the total output of sugar was below the standard epinephrin excretion in one instance and much above it in the other. If 3 or more hours are allowed to elapse between the introduction of sodium phosphate and that of epinephrin (Experiments 43, 45, and 50) the blood sugar curve is much less elevated and prolonged than that provoked by epinephrin alone, and the average sugar excretion is much below the standard output induced by epinephrin. It is therefore apparent that injections of sodium phosphate will markedly diminish and shorten the period of hyperglycemia caused by epinephrin and will also appreciably decrease the elimination of sugar in the urine.

#### SUMMARY:

Under the experimental conditions subcutaneous injections of calcium chloride and calcium lactate fail to induce significant changes in the blood sugar content of normal rabbits.

Subcutaneous administration of calcium salts previous to epinephrin introduction may provoke distinct alterations in the character of the curve of epinephrin hyperglycemia and always increase the output of urinary sugar. The time relation between the injection of calcium and of epinephrin and the quantity of calcium salt given constitute the factors which determine the degree of deviation which the curve of blood sugar content may assume. In general, the most marked effect is obtained, both upon the epinephrin hyperglycemia curve and the excretion of sugar, if 10 cc. of 1 to 3 per cent calcium lactate solution are introduced 2 hours previous to the injection of epinephrin.

The subcutaneous or intravenous injection of trisodium phosphate may cause, although not invariably, a distinct diminution in the blood sugar content of normal rabbits. The subcutaneous administration of sodium oxalate may call forth a similar influence, although the condition may be first preceded by an augmented blood sugar content. It is probable that this action of these salts is attributable to their common property of combining with calcium.

Given subcutaneously to rabbits under suitable conditions trisodium phosphate will cause a marked lowering and shortening

of the curve of epinephrin hyperglycemia and the excretion of sugar in the urine may be decreased significantly.

#### CONCLUSIONS.

*Calcium salts play a noteworthy rôle in the regulation of blood sugar content*, for (1) although an increase of calcium is without marked effect in normal animals such an augmentation in rabbits with a disturbed carbohydrate metabolism, in epinephrin hyperglycemia, may result in a distinct change in the character of the curve of epinephrin hyperglycemia and may also cause a noticeably increased elimination of sugar in the urine; (2) withdrawal of calcium by introduction of sodium phosphate, for example, will produce in normal rabbits, although not invariably, a condition of hypoglycemia; and (3) the injection of epinephrin at an optimum period after sodium phosphate introduction produces a briefer period of hyperglycemia with a lower sugar level than that yielded by epinephrin alone. The sugar elimination by the kidney may also be less than the average output under epinephrin only.





## STUDIES IN CARBOHYDRATE METABOLISM.

### XII. THE INFLUENCE OF SODIUM CARBONATE UPON BLOOD SUGAR CONTENT AND UPON EPINEPHRIN HYPERGLYCEMIA AND GLYCOSURIA.\*

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Under normal conditions the blood sugar content may be accepted as constituting one of the body constants. The exact mechanism whereby this regulation is maintained is unknown. The introduction into the organism of various types of substances, such as epinephrin, phlorhizin, phosphorus, hydrazine, etc., is succeeded by a temporary failure of the blood sugar-regulating apparatus which may be manifested by the development of hyperglycemia with glycosuria or by a condition of hypoglycemia in which sugar may or may not appear in the urine. The equilibrium of the blood sugar-regulating mechanism is undoubtedly exceedingly sensitive to a great variety of chemical influences. This is particularly true with respect to circumstances which tend toward the production of a condition of acidosis, as exemplified by the hyperglycemia and glycosuria which may be evoked by dyspnea and allied states. That acidosis exerts a distinct influence upon carbohydrate metabolism is attested by the observations of Elias,<sup>1</sup> who demonstrated that the introduction of hydrochloric acid into dogs and rabbits leads to hyperglycemia and glycosuria. Elias<sup>2</sup> has also shown that the so called hunger diabetes<sup>3</sup> of young dogs is, in part at least, a condition due to

\* Reported at the December meeting of the American Society for Biological Chemists, Boston, 1915. See also *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 111.

<sup>1</sup> Elias, H., *Biochem. Z.*, 1912-13, xlviii, 120.

<sup>2</sup> Elias, H., and Kolb, L., *Biochem. Z.*, 1913, lii, 331.

<sup>3</sup> Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1889-90, xxvi, 355.

acidosis, as indicated by the carbon dioxide content of the blood and analysis of the alveolar air. Observations upon human diabetes also teach that in this condition acidosis may play a part. From this brief review it is evident that the introduction of acid or the production of acid in the organism is associated with a disturbance of blood sugar content and is manifested in one direction; namely, blood sugar content is augmented. On the other hand, the influence of a state of alkalosis upon blood sugar content has received little or no attention.

While attempting to demonstrate the relation of calcium to blood sugar content, as reported in the preceding paper, it was observed that the introduction of alkali into the blood stream sometimes caused a marked diminution in the sugar of the blood. In the present communication are given the results of these experiments together with those designed to test the influence of alkali upon epinephrin hyperglycemia and glycosuria.

### *Methods.*

The general methods employed were those outlined in the preceding article. Well fed, full grown rabbits received into the ear vein injections of sodium carbonate solutions, the period of introduction varying from 2 to 5 minutes. Anesthesia was never resorted to. Usually the only symptom observable was a greatly diminished respiration and noticeable peristalsis. Blood for analysis was withdrawn from the ear vein, sugar being estimated by the Forschbach and Severin method. Adrenalin (Parke, Davis and Company 1 : 1,000) was introduced subcutaneously in the dosage of 1 cc. per kilo of body weight. Sugar in the urine was estimated by the Allihn procedure.

### *The Influence of Intravenous Injections of Sodium Carbonate upon Blood Sugar Content.*

That alkali introduction exerts an action upon carbohydrate regulation in the body was demonstrated by Pavy and Godden,<sup>4</sup> who found that the glycosuria provoked by ether or chloroform disappears after the intravenous injection of sodium carbonate. Given by mouth or intravenously sodium carbonate will abolish the hyperglycemia of hunger diabetes, and the glycosuria, according to Elias,<sup>2</sup> will either entirely disappear or be greatly dimin-

<sup>4</sup> Pavy, F. W., and Godden, W., *J. Physiol.*, 1911-12, xliii, p. vii.

ished. After removal of the pancreas, sodium carbonate introduced into the blood stream of the dog causes a diminution of the sugar excretion.<sup>5</sup> Later work by Murlin and Kramer<sup>6</sup> has shown that under the influence of sodium hydroxide the respiratory quotient is increased in depancreatized dogs. In agreement with this finding are the results of Kramer and Marker<sup>7</sup> who have stated that, "Sugar retained when sodium carbonate is administered to depancreatized dogs is not stored as glycogen nor is it eliminated by way of the saliva or the gastro-intestinal tract." These observations make it evident that a condition of alkalosis may exert an influence upon carbohydrate metabolism, but this effect is manifested by retention of carbohydrate within the organism.

The explanation for the closely agreeing observations just cited probably lies in the effect which sodium carbonate exerts at times upon blood sugar content. This influence may be seen by reference to Table I. It is evident from these figures that the intravenous administration of sodium carbonate will cause a marked diminution in the blood sugar content of normal rabbits, but that this effect is not invariable. It will be observed that the quantity of alkali injected bears little relation to the action exerted upon blood sugar content. Expressed differently, one may say that the introduction of approximately 0.5 gm. of sodium carbonate is just as efficacious when dissolved in 100 cc. of water as it is in stronger concentration or even in greater absolute amount. Blood sugar content is not always<sup>8</sup> affected but when there is a diminution it is transient, the normal being regained usually within 1 or 1½ hours. If a second injection is given at a period when blood sugar is still low, the interval of hypoglycemia may be lengthened noticeably (Rabbit 92). The diminution in the percentage of blood sugar cannot be ascribed to mere dilution of the blood by the introduced fluid, because it has been found by experiments in this laboratory<sup>9</sup> that under the conditions

<sup>5</sup> Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1913, xv, 365.

<sup>6</sup> Murlin and Kramer, *J. Biol. Chem.*, 1916, xxiv, p. xxv.

<sup>7</sup> Kramer, B., and Marker, J., *J. Biol. Chem.*, 1916, xxiv, p. xxiv.

<sup>8</sup> The possible reasons for the irregularities in this respect are being investigated.

<sup>9</sup> To be published shortly.

TABLE I.

*The Influence of Intravenous Injections of Sodium Carbonate upon Blood Sugar Content.*

Rabbit.	Body weight	Blood sugar content (in percentages).							Remarks.
		Normal	Hours after carbonate injection.						
			$\frac{1}{2}$	1	$1\frac{1}{2}$	2	3	4	
	gm.								Injection of 0.4 per cent $\text{Na}_2\text{CO}_3$ .
82	2,500	0 15		0 05		0.11	0.09	0 11	100 cc.
83	2,000	0 14		0 05		0 12	0.13	0.11	80 "
84	2,100	0 12	0 05	0 04	0 10	0 09			} 10.20 a.m., 100 cc. 2.30 p.m., 100 "
		0 15	0 03	0 03	0 02	0 11			
85	2,000	0 12	0 02	0 05	0.12	0 15			} 10.20 a.m., 100 " 2.30 p.m., 100 "
		0 15	0 03	0 03	0 02	0 11			
94	2,000	0 17	0 16	0 11					100 cc.
95	2,000	0 16	0 12	0 09					100 "
99	2,000	0 18	0 13	0 12		0 17	0.15		120 "
									Injection of 1.0 per cent $\text{Na}_2\text{CO}_3$ .
102	1,600	0 11	0 18	0 15		0 18	0.16		} 10.30 a.m., 80 cc. 2.30 p.m., 80 "
		0 16	0 14	0 13		0 12			
103	1,600	0 16	0 14	0 13		0 12	0.12		} 10.30 a.m., 80 " 2.30 p.m., 80 "
		0 12	0 12	0 13		0 14			
									Injection of 2.0 per cent $\text{Na}_2\text{CO}_3$ .
92	1,600	0 13	0 05	0 04	0 04	0.05	0 04	0.14	{ 10.30 a.m., 30 cc. 11.30 " 20 "
93	1,600	0 13	0 02	0 05					
									30 cc. Killed by gas bubble during second injection.

of the present experiments blood volume is restored to normal within a period of 20 to 25 minutes.

The regulation of blood sugar content would appear to be associated in part at least with the equilibrium between acids and bases in the body. Acid tends to increase blood sugar content; it has an influence in causing sugar elimination. On the other hand, base or alkali tends to reduce the blood sugar content. These ideas fit in well with the facts known concerning the relation of thyreoparathyroidectomy to sugar metabolism. It has been shown by Underhill and Blatherwick<sup>10</sup> that previous

<sup>10</sup> Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 67. .... 110

to the tetany provoked by removal of the thyroid-parathyroid mechanism, blood sugar content may be greatly diminished. Calcium relieves the tetany and restores blood sugar to normal. In the preceding paper of this series it has been demonstrated that under disturbed conditions of carbohydrate metabolism calcium behaves in a manner analogous to hydrochloric acid. Wilson and his coworkers<sup>11</sup> have found that previous to the onset of tetany after removal of the thyroids and parathyroids a condition of alkalosis obtains. The introduction of hydrochloric acid relieves the symptoms equally as well as calcium administration. The period of alkalosis corresponds fairly well with the interval of low blood sugar observed by Underhill and Blatherwick. It is probable, therefore, that the condition of alkalosis may be responsible for the hypoglycemia. The resumption of normal blood sugar is induced by either calcium or hydrochloric acid which have a common behavior in increasing blood sugar content under suitable conditions.

*The Effect of Intravenous Injections of Sodium Carbonate upon Epinephrin Hyperglycemia and Glycosuria.*

Both calcium and hydrochloric acid cause a marked augmentation of epinephrin hyperglycemia and glycosuria. In view of the fact that sodium carbonate may bring about a condition of hypoglycemia in normal rabbits and decrease the hyperglycemia in dogs after pancreas removal, it seemed desirable to determine the action of sodium carbonate upon the hyperglycemia and glycosuria induced by epinephrin. This has been done and the results obtained are detailed in Table II.

When sodium carbonate is introduced into the blood stream in quantities above 0.5 gm. at an interval of  $\frac{1}{2}$  to 1 hour previous to epinephrin administration, there is a marked influence upon both the hyperglycemia and glycosuria. The hyperglycemia is distinctly lower and its period of duration decidedly shorter than when the sodium carbonate has been omitted.<sup>12</sup> Likewise the

<sup>11</sup> Wilson, D. W., Stearns, T., and Thurlow, M. D., *J. Biol. Chem.*, 1915, xxiii, 100. Wilson, D. W., Stearns, T., and Janney, J. H., Jr., *ibid.*, 1915, xxiii, 123.

<sup>12</sup> See preceding paper for hyperglycemia curve.

TABLE II.

The Influence of Intravenous Injections of Sodium Carbonate upon Epinephrin Hyperglycemia and Glycosuria.

Rabbit	Body weight	Blood sugar content (in percentages)														Sugar in urine	Remarks.
		Hours after injection of															
		Normal	Epinephrin.														
			Sodium carbonate														
				½	1	1½	2	2½	3	3½	4	4½	5	5½			
gm.	½	1	1½	2	2½	3	3½	4	4½	5	5½	gm					
86	2,400	0.12	0.02	0.01	0.11	0.23	0.18	0.15	0.16				0.54	Injection of 0.4 per cent Na <sub>2</sub> CO <sub>3</sub> . 100 cc + epinephrin.			
					0.26	0.27							0.72	Epinephrin alone 5 hours after first injection.			
87	1,800	0.12	0.02	0.05	0.15	0.15	0.12	0.16					0.00	80 cc. + epinephrin.			
					0.27	0.24							0.83	Epinephrin alone 5 hours after first injection.			
90	1,800	0.12	0.13		0.13	0.18	0.30	0.07	0.06				0.20	Injection of 2.0 per cent Na <sub>2</sub> CO <sub>3</sub> . 30 cc.			
91	2,200	0.11	0.12		0.11	0.26	0.24	0.19	0.15				0.75	35 "			
106	2,200	0.15	0.12		0.21	0.30		0.31	0.24	0.13			2.24	Injection of 1.0 per cent Na <sub>2</sub> CO <sub>3</sub> 75 cc.			
107	2,200	0.11	0.10		0.15	0.23	0.26	0.24	0.15	0.14			0.76	100 "			
108	2,200	0.13	0.13		0.10	0.16	0.26	0.25	0.25	0.19			0.48	125 "			
109	2,100	0.13	0.15		0.16	0.16	0.21	0.29	0.30	0.12			0.26	125 "			
88	1,600	0.11			0.27	0.27	0.10	0.20	0.16	0.13			2.13	Injection of 0.4 per cent Na <sub>2</sub> CO <sub>3</sub> and epinephrin simultaneously.			
89	2,000	0.12			0.19	0.29	0.26	0.23	0.16	0.17			2.10	75 cc 100 "			

sugar elimination in general falls far below the average excretion observed when only epinephrin has been injected. In one instance (Rabbit S7) neither increased blood sugar nor glycosuria was in evidence. That this animal was not glycogen-free is proved by the fact that a second injection of epinephrin on the same day called forth an excretion of an appreciable quantity of sugar. Evidently it is not essential that there be an initial drop in blood sugar previous to epinephrin injection in order to obtain the typical influence upon epinephrin hyperglycemia and glycosuria. This may be seen readily from Rabbits 90 to 109 inclusive. It is essential, however, that at least  $\frac{1}{2}$  hour should elapse between the administration of sodium carbonate and that of epinephrin, as may be seen from the results with Rabbits 88 and 89 where the sodium carbonate and epinephrin were given nearly simultaneously. The extent and duration of hyperglycemia<sup>12</sup> more nearly resemble those observed with epinephrin alone. Even in this case, however, the duration of hyperglycemia has been significantly decreased. Glycosuria was also very much greater in both cases than in any of those, with a single exception, where an appreciable time interval occurred between the two injections.

#### CONCLUSIONS.

The intravenous injections of sodium carbonate into rabbits may induce a marked though transient fall in blood sugar content.

It is suggested that the acid-base equilibrium is a factor in blood sugar regulation.

The significance of this view in its relation to carbohydrate metabolism after thyreoparathyroidectomy is indicated.

The hyperglycemia and glycosuria provoked by epinephrin are both significantly decreased if sodium carbonate is administered at suitable periods of time previous to epinephrin introduction.





## STUDIES IN CARBOHYDRATE METABOLISM.

### XIII. THE INFLUENCE OF MAGNESIUM SALTS UPON BLOOD SUGAR CONTENT AND UPON EPINEPHRIN HYPERGLYCEMIA AND GLYCOSURIA.

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In a previous communication<sup>1</sup> it has been pointed out that under suitable conditions calcium may exert a significant influence upon certain phases of carbohydrate metabolism. Since it is generally assumed that calcium and magnesium within the organism may play antagonistic rôles it seemed desirable to investigate more closely than has been done previously the relationship of magnesium to blood sugar content both under normal conditions and in hyperglycemia experimentally produced. Our knowledge of the possible influence of magnesium upon carbohydrate metabolism is slight. Meltzer and Auer,<sup>2</sup> during the course of an investigation on the general behavior of magnesium salts in the body, incidentally observed the appearance of sugar in the urine. Underhill and Closson<sup>3</sup> who studied the phenomenon more closely showed that the intravenous injection of magnesium sulfate caused a significant hyperglycemia which they ascribed to disturbances of respiration, dyspnea, induced by the administration of magnesium. A recent report<sup>4</sup> by Kleiner and Meltzer confirms these results, although the latter investigators believe that the condition of hyperglycemia is evoked in some specific manner by the magnesium salt. Airila and Bardy<sup>5</sup> state

<sup>1</sup> Underhill, F. P., *J. Biol. Chem.*, 1916, xxv, 447.

<sup>2</sup> Meltzer, S. J., and Auer, J., *Am. J. Physiol.*, 1905-06, xiv, 371.

<sup>3</sup> Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905-06, xv, 321.

<sup>4</sup> Kleiner, I. S., and Meltzer, S. J., *J. Biol. Chem.*, 1916, xxiv, p. xx.

<sup>5</sup> Airila, Y., and Bardy, H., *Skand. Arch. Physiol.*, 1915, xxxii, 246.

that the intravenous injection of magnesium salts diminishes or inhibits the glycosuria produced by epinephrin.

The present investigation was planned to determine the influence of (a) small doses of magnesium upon blood sugar content in normal animals, (b) small doses of magnesium upon epinephrin hyperglycemia and glycosuria, (c) anesthetic doses of magnesium salts upon blood sugar content and upon epinephrin hyperglycemia and glycosuria, and (d) the influence of calcium upon the extent and character of the hyperglycemia and glycosuria provoked by magnesium salts.

### Methods.

Rabbits were employed throughout. Estimation of sugar in the blood and urine was made in accordance with the procedures cited in a previous paper.<sup>1</sup> Magnesium was injected subcutaneously and calcium was introduced through the marginal ear vein. The epinephrin used was adrenalin of Parke, Davis and Company 1 : 1,000, 1 cc. or 1 mg. per kilo of body weight constituting the dosage employed.

### *The Influence of Subcutaneous Injections of Magnesium Lactate upon Blood Sugar Content and upon Epinephrin Hyperglycemia and Glycosuria.*

It may be seen from an inspection of Table I that in the doses employed a 3 per cent solution of magnesium is without influence upon the blood sugar content of normal animals during a period of 6 hours. The introduction of magnesium under the experimental conditions is attended by no symptoms.

TABLE I.  
*The Influence of Magnesium Lactate upon Blood Sugar Content.*

Rabbit.	Body weight	Blood sugar content (in percentages).						Subcutaneous injection of 3 per cent solution of magnesium lactate.	
		Normal.	Hours after injection of magnesium lactate.						
			1	2	3	4	5		6
	gm							cc.	
54	2,000	0 16	0 15	0 14	0 13	0 11	0 12	0 10	10
55	2,000	0 13	0 15	0 12	0 14	0 14	0 10	0 10	10
56	2,000	0 13	0 15	0 16	0 13	0 12	0 13	0 15	20
57	2,000	0 11	0 11	0 12	0 14	0 11	0 14	0 12	20

On the other hand, if comparable doses of magnesium are given to rabbits at various periods previous to epinephrin administration a distinct influence may be observed both upon the curve of hyperglycemia and upon the sugar elimination by the kidney (Table II). The effect upon hyperglycemia is manifested chiefly by a significant shortening of the duration of this condition, the maximum of the hyperglycemia not being influenced appreciably. The maximum effect upon hyperglycemia appears to be produced when the magnesium salt has been introduced 1 or 2 hours previous to the injection of epinephrin. A distinctly lower level and shorter duration of hyperglycemia are to be noted when the epinephrin has been administered 3 hours after the introduction of magnesium. Magnesium lac-

TABLE II.

*The Influence of Magnesium Lactate upon Epinephrin Hyperglycemia and Glycosuria.*

Rabbit.	Body weight	Blood sugar content (in percentages)										Sugar in urine	Subcutaneous injection of 3 per cent solution of magnesium lactate	
		Normal	Hours after injection of											
			Magnesium lactate			Epinephrin								
			1	2	3	1	2	3	4	5	6			
	gm.											gm	cc	
58	2,000	0 14	0 13	0 12		0 34	0 34	0 40	0 39	0 28		3 58	20	
59	2,000	0 14	0 14	0 14	0 14	0 33	0 40	0 35	0 30	0 25		7 72		
60	2,000	0 18	0 13	0 14		0 33	0 47	0 42	0 30	0 23	0 14	2 85		
61	1,800	0 17	0 11	0 13		0 32	0 50	0 41	0 30	0 29	0 09	2 52		
Average						0 33	0 43	0 39	0 32	0 26	0 11	4 17		
62	1,800	0 14	0 15			0 37	0 41	0 36	0 21	0 19	0 15	2 96	20	
63	3,000	0 13	0 12			0 39	0 39	0 27	0 24	0 20	0 12	3 60	30	
Average.						0 38	0 40	0 32	0 23	0 20	0 14	3 28		
64	2,200	0 18	0 17	0 18	0 19	0 33	0 33	0 23	0 23	0 18		2 48	30	
65	1,800	0 16	0 17	0 17	0 17	0 21	0 33	0 25	0 22	0 17		1 05	20	
66	1,800	0 15			0 17	0 25	0 33	0 32	0 21	0 17		3 15	20	
67	1,800	0 14			0 14	0 28	0 35	0 34	0 22	0 17		1 70	20	
Average						0 27	0 33	0 28	0 22	0 17		2 09		

tate seems to favor the elimination of sugar by the kidney during epinephrin hyperglycemia, for the average amount of sugar appearing in the urine is greater when magnesium injection precedes epinephrin introduction than it is when epinephrin is given alone.<sup>1</sup> The maximum effect in this respect is seen if magnesium lactate is injected 2 hours previous to epinephrin.

This influence of magnesium is still manifested if the magnesium injection precedes that of epinephrin by 1 hour. Epinephrin introduced 3 hours after magnesium produces a greater average quantity of sugar than is found after epinephrin administration only, but the quantity of sugar excreted is decidedly less than is called forth by the introduction of epinephrin at periods 1 or 2 hours after magnesium.

Under the experimental conditions magnesium lactate appears to exert an influence upon carbohydrate metabolism similar to that evoked by calcium; that is, both induce a greater output of sugar in epinephrin glycosuria.

*The Effect of Anesthetic Doses of Magnesium Sulfate upon Blood Sugar Content and upon Epinephrin Hyperglycemia and Glycosuria.*

The results outlined in Table III make it evident that doses of magnesium sulfate too small to produce anesthesia have only a slight transitory influence upon blood sugar content in normal rabbits. This slight effect is in the direction of inducing hyperglycemia, but the degree of high blood sugar content is insufficient to elicit the appearance of sugar in the urine. When, however, magnesium sulfate is injected in quantities leading to anesthesia hyperglycemia is marked and may continue for several hours. Under these circumstances the urine volume is low and the total quantity of sugar excreted is very small.

The effect of anesthetic doses of magnesium sulfate upon epinephrin hyperglycemia and glycosuria may be seen in the experiments detailed in Table IV. It is obvious that under the experimental conditions magnesium does not greatly alter the height or duration of epinephrin hyperglycemia nor is the output of sugar through the urine profoundly changed from that by epinephrin alone.

TABLE III

*The Influence of Anesthetic Doses of Magnesium Sulfate upon Blood Sugar Content*

Rabbit	Body weight	Blood sugar content (in percentages)									Sugar in urine	Subcutaneous injection of 2% $MgSO_4$
		Normal	Hours after injection of magnesium sulfate									
			1	2	3	4	5	6	7			
	gm										gm	cc
68	2,300	0 17	0 18	0 22	0 20	0 14	0 14	0 13	0 13	0		15*
69	3,800	0 15	0 19	0 18	0 17	0 11	0 12	0 13	0 12	0		20*
72	1,900	0 17	0 25	0 37	0 33	0 33	0 25	0 16	0 18	0 80		25†
73	2,200	0 15	0 31	0 48	0 47	0 35	0 22	0 22	0 16	0 36		30†
74	2,100	0 15	0 29	0 40	0 21	0 15				0 65		25†
75	2,000	0 16	0 19	0 29	0 41	0 31	0 19	0 15		0 34		25†

\* No anesthesia

† Complete anesthesia

TABLE IV

*The Influence of Anesthetic Doses of Magnesium Sulfate upon Epinephrin Hyperglycemia and Glycosuria*

Rabbit	Body weight  <i>gm</i>	Blood sugar content (in percentages)										Sugar in urine  <i>gm</i>	Remarks
		Normal	Hours after injection of										
			Magnesium sulfate		Epinephrin								
1	2	1	2	3	4	5	6						
70	2,800	0 18	0 20	0 28	0 42	0 42	0 37	0 37	0 33	0 23	2 16	Subcutaneous injection of 2N MgSO <sub>4</sub> , 30 cc Deep anesthesia in 1 hour	
71	2,200	0 18	0 29	0 31	0 37	0 37	0 36	0 36	0 36	0 34	1 89		

*The Influence of Intravenous Injections of Calcium Chloride upon the Hyperglycemia and Glycosuria Provoked by Magnesium Sulfate.*

Meltzer and Auer<sup>6</sup> have shown that an animal anesthetized by an injection of magnesium sulfate may be quickly restored to a normal state by the intravenous administration of calcium

<sup>6</sup> Meltzer and Auer, *Am J Physiol*, 1908, **vii**, 400

chloride. Since the hyperglycemia and glycosuria subsequent to magnesium injection are most evident during the period of anesthesia it was of interest to determine what influence would be exerted upon blood sugar content by the recovery from anesthesia through calcium administration. The results of four such experiments may be seen in Table V. In each instance the rabbit

TABLE V.

*The Influence of Calcium Chloride upon the Hyperglycemia and Glycosuria Provoked by Magnesium Sulfate.*

Rabbit	Body weight	Blood sugar content (in percentages).				Sugar in urine.	Remarks.
		Normal	Hours after injection of				
			Magnesium sulfate.	Calcium chloride			
				1	1		
	gm						
78	2,400	0 13	0 16	0 26	0 13	0	Subcutaneous injection of 25 cc. 2x MgSO <sub>4</sub> . Complete anesthesia resulted within an hour. 1 hour after the MgSO <sub>4</sub> injection each rabbit received 10 cc. 2.5 per cent solution of CaCl <sub>2</sub> intravenously. Within 2 minutes the animal had entirely recovered from the deep anesthesia and was sitting in a normal position.
79	2,400	0 13	0 19	0 22	0.14	Trace.	
80	2,100	0 12	0 19	0 10	0 11	0	
81	2,100	0 14	0 20	0 13	0 10	0	

was in a state of profound anesthesia within 1 hour after magnesium injection. The range of blood sugar content usually obtained with magnesium anesthesia may be found in Table III. Comparison of these figures with those of Table V shows that the introduction of calcium caused a rapid reduction of the hyperglycemia produced by magnesium to normal blood sugar content, this effect being manifested within an hour or two. Glycosuria either entirely failed to develop or else was present to a slight extent only.

Inasmuch as it has been shown that calcium salts and hydrochloric acid produce similar types of influence upon blood sugar content during epinephrin hyperglycemia and glycosuria and since calcium chloride has an antagonistic action to magnesium with respect to anesthesia, it seemed desirable to test the influence of hydrochloric acid upon the state of anesthesia evoked by magnesium sulfate. For this purpose a rabbit of 2,200 gm. body weight received an injection of 30 cc. 2 N magnesium sulfate subcutaneously. Deep anesthesia developed in 45 minutes. Intravenous administration of 20 cc. 0.1 per cent hydrochloric acid solution in 3 minutes caused accelerated respiration and return of the eye reflex temporarily, but the conditions peculiar to magnesium anesthesia were soon resumed. A second rabbit of 2,200 gm. body weight was given a subcutaneous injection of 30 cc. 2 N magnesium sulfate solution which was followed by the usual deep anesthesia. The intravenous injection of 20 cc. 1.0 per cent hydrochloric acid solution caused only an accelerated respiration. The influence of alkali introduction was tested upon a third rabbit weighing 3,000 gm. Anesthesia was induced by the subcutaneous injection of 30 cc. 2 N magnesium sulfate solution. The intravenous administration of 60 cc. 1.0 per cent sodium carbonate solution was without noticeable effect.

It is apparent, therefore, that administration of hydrochloric acid or sodium carbonate is without significant influence upon magnesium anesthesia.

#### SUMMARY.

Small doses of magnesium lactate introduced subcutaneously fail to produce significant changes in the blood sugar content of the rabbit.

The same dosage of magnesium lactate injected in like manner intensifies the effect of epinephrin upon blood sugar content and glycosuria. The maximum influence is seen when the magnesium salt is given 2 hours previous to epinephrin treatment.

A significant hyperglycemia is produced by subcutaneous administration of magnesium sulfate if general anesthesia develops. Glycosuria is always slight. If anesthesia fails to develop, hyperglycemia is not pronounced and is of short duration. Under these conditions sugar fails to appear in the urine.



Injection of calcium during the height of magnesium anesthesia quickly abolishes this state, as demonstrated by Meltzer and Auer, and in correspondence with this antagonism there is a rapid return of blood sugar content to the normal level. Glycosuria is either absent or is present to a small degree only.

Administration of hydrochloric acid or sodium carbonate is without significant influence upon magnesium anesthesia.

# HYDROGEN ELECTRODE POTENTIALS OF PHTHALATE, PHOSPHATE, AND BORATE BUFFER MIXTURES.\*

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## INTRODUCTION.

At the present stage of its development the colorimetric method of determining hydrogen ion concentration requires the use of a set of comparison solutions whose hydrogen ion concentrations have been accurately determined by hydrogen electrode measurements. Of the various sets which have been proposed the most complete and reliable is that of Sorensen (35, 36). This set may be supplemented by various other mixtures among which should be mentioned the acetic acid-sodium acetate mixtures carefully studied by Walpole (38), cacodylate mixtures (30, 37), and Palitzsch's (33) boric acid-borate mixtures.

The present study will add to the available list the phthalate mixtures which received their first brief treatment by Fels (16).

We have also studied a selection of mixtures which as a set seem to us to possess certain advantages over those in common use. In our selection we have been guided largely by the desire to simplify the technique of preparation and to reduce the number of substances required.

We have considered it advisable to eliminate as far as possible the use of substances which crystallize with water of crystallization. Citric acid used in the Sørensen mixtures crystallizes with water of crystallization, and it effloresces so easily that dehydration or else determination of the water content is necessary. Complete elimination of the water without decomposition of the

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acid is a delicate operation. Furthermore, citric acid sometimes crystallizes anhydrous, a fact which might easily cause confusion. We also have reason to believe that in the preparation of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  from  $\text{Na}_2\text{HPO}_4 \cdot 10\text{H}_2\text{O}$  by Sørensen's method some workers have not appreciated the conditions or else the length of exposure necessary.<sup>1</sup> Our objection to the use of substances which crystallize with water of crystallization applies essentially to a substance we have had to retain—boric acid. Boric acid is ordinarily considered to crystallize without water of crystallization inasmuch as it precipitates with a composition which may be expressed as  $\text{H}_3\text{BO}_3$ ; nevertheless, it loses water at a comparatively low temperature and is reported to change to metaboric acid between 80 and 100°. We have had no difficulty, however, in obtaining air-dried boric acid which loses less than 0.1 per cent in weight when held for a week over calcium chloride. If we are to believe Berzelius (3) (1835) and the statements frequently made since his observation, borax, if carelessly handled, might become seriously impregnated with carbonate. The same might be true of disodium phosphate. Considerations such as these have led us to reduce the number of alkaline substances and alkaline stock solutions in our set to one.

The acetic acid in the acetate mixtures is volatile and requires somewhat elaborate purification. The acetate mixtures are furthermore prone to support mold growth, although not to the extent which makes glyocoll so troublesome in standard solutions.

The following is the set of mixtures we propose:

Acid potassium <i>o</i> -phthalate—hydrochloric acid.	
“ “ “ —sodium hydroxide.	
“ “ phosphate — “ “	
Boric acid + KCl — “ “	

It will be noted that all the solid substances crystallize beautifully from their aqueous solutions without water of crystallization. All but the boric acid may be dried at 110°C. in an ordinary oven. The boric acid may be dried fairly well in bulk by exposing it to the air, on a dry day, and can be spread finally in thin layers

<sup>1</sup> We have observed a 10 per cent increase in the weight of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  exposed to a humid atmosphere. This is unlikely to occur under ordinary laboratory conditions but may be serious in humid climates.

in a desiccator over calcium chloride. Only the sodium hydroxide need be protected from the  $\text{CO}_2$  of the atmosphere.

The proper preparation of the sodium hydroxide solution may appear to be a difficult matter. Later we shall outline a method of preparation which furnishes a solution sufficiently pure for the present purpose.

Fig. 1, which must be drawn to a larger scale from the data given later before it can be used as is Sørensen's chart (36), will serve to show that the curve of the phthalates overlaps the curve of the phosphates sufficiently to allow the preparation of phthalate and phosphate solutions of the same hydrogen ion concentration. In like manner the phosphate and borate curves overlap. This is important, for it makes it possible to check colorimetrically the consistency of any particular set of these solutions. It will also be seen that the phthalate-HCl mixtures run up into the region which may be covered by HCl-KCl mixtures, and, were these added to the list, the entire range of hydrogen ion concentration of any physiological importance could be covered. Some preliminary measurements of KCl-HCl mixtures at  $25^\circ$  are given in Table VII.

Acid potassium phthalate, as shown by Dodge (14) and confirmed by Hendrixson (19), is an excellent substance for the standardization of alkali solutions. We therefore have within the set a substance which may be relied upon to standardize the sodium hydroxide solution and indirectly the hydrochloric acid solution. The other substances can be weighed accurately.

We shall describe in the following pages the electrometric measurements made, and shall discuss the values of diffusion and calomel electrode potentials used in the calculation of the hydrogen electrode potentials and  $P_H$  values of these mixtures.

### *Apparatus and Methods.*

*General Procedure.*—The hydrogen electrode measurements were made with the chain: Hg,  $\text{HgCl}$ ,  $\text{KCl}$  (0.1 N) /  $\text{KCl}$  (saturated) / Solution, Pt,  $\text{H}_2$ . Instead of measuring the potential of this chain directly, it was measured in two steps. First, the chain Hg,  $\text{HgCl}$ ,  $\text{KCl}$  (saturated) / Solution, Pt,  $\text{H}_2$  was measured. The "saturated calomel electrode" was used as recommended by

acid is a delicate operation. Furthermore, citric acid sometimes crystallizes anhydrous, a fact which might easily cause confusion. We also have reason to believe that in the preparation of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  from  $\text{Na}_2\text{HPO}_4 \cdot 10\text{H}_2\text{O}$  by Sørensen's method some workers have not appreciated the conditions or else the length of exposure necessary.<sup>1</sup> Our objection to the use of substances which crystallize with water of crystallization applies essentially to a substance we have had to retain—boric acid. Boric acid is ordinarily considered to crystallize without water of crystallization inasmuch as it precipitates with a composition which may be expressed as  $\text{H}_3\text{BO}_3$ ; nevertheless, it loses water at a comparatively low temperature and is reported to change to metaboric acid between 80 and 100°. We have had no difficulty, however, in obtaining air-dried boric acid which loses less than 0.1 per cent in weight when held for a week over calcium chloride. If we are to believe Berzelius (3) (1835) and the statements frequently made since his observation, borax, if carelessly handled, might become seriously impregnated with carbonate. The same might be true of disodium phosphate. Considerations such as these have led us to reduce the number of alkaline substances and alkaline stock solutions in our set to one.

The acetic acid in the acetate mixtures is volatile and requires somewhat elaborate purification. The acetate mixtures are furthermore prone to support mold growth, although not to the extent which makes glycocoll so troublesome in standard solutions.

The following is the set of mixtures we propose:

Acid	potassium	<i>o</i> -phthalate	—	hydrochloric acid.
"	"	"	—	sodium hydroxide.
"	"	phosphate	—	" "
Boric acid	+	KCl	—	" "

It will be noted that all the solid substances crystallize beautifully from their aqueous solutions without water of crystallization. All but the boric acid may be dried at 110°C. in an ordinary oven. The boric acid may be dried fairly well in bulk by exposing it to the air, on a dry day, and can be spread finally in thin layers

<sup>1</sup> We have observed a 10 per cent increase in the weight of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  exposed to a humid atmosphere. This is unlikely to occur under ordinary laboratory conditions but may be serious in humid climates.

Michaelis and Davidoff (29), not only because it requires less careful protection from the adjacent concentrated potassium chloride solution, but because its low electrical resistance permits a fuller use of the galvanometer's sensitivity. Since such an electrode is by no means as constant as the 0.1 N calomel electrode the electrode used was connected with five similar electrodes so that drifts of potential could be detected. The second step consisted in measuring the potential of the saturated electrode against the average of six 0.1 N calomel electrodes.

The hydrogen electrode vessels were the type described previously by one of us (8). Before each measurement, the vessel was thoroughly rinsed with several portions of the solution to be tested, and a vigorous stream of hydrogen was then bubbled through the solution in the vessel. The hydrogen bearing upon the solution was then brought to atmospheric pressure, the drain-cock was closed, and the vessel shaken from 10 to 15 minutes. Shaking was then stopped and the liquid contact with the saturated potassium chloride solution was made. The potential was measured immediately with the electrode fully immersed. This first-measured potential was always taken as the significant value. Readings were continued for 5 minutes in order to make sure that any drift which might occur was small and regular. A duplicate determination was then made with a second vessel and second electrode. If, as seldom happened, the two determinations were more than 0.05 millivolt apart, measurements were repeated until a series of consistent values were obtained.

*Diffusion Potentials.*—The diffusion potential between saturated and 0.1 N KCl solutions was neglected. Therefore, the potential of the electrode described as Hg, HgCl, KCl (0.1 N) is really the half cell Hg, HgCl, KCl (0.1 N) / Saturated KCl.

The diffusion potential at the liquid contact with the solution in the hydrogen electrode vessel was reduced by making this contact with saturated potassium chloride solution. The Bjerrum (4, 5) extrapolation was found by essentially the same method employed by Walpole (38) in his studies of the acetic acid-sodium acetate mixtures.

Since Bjerrum himself showed that this extrapolation does not furnish values in perfect accord with those deduced from theory, and developed a theoretical explanation of why they could not,

and since Bjerrum and others have shown that the observed diffusion potentials are to some extent a function of the manner of the formation of the liquid juncture, we regard our extrapolations merely as indicating the order of magnitude of the correction to be applied.

*Calomel Electrodes.*—The mercury used in the calomel electrodes was thrice distilled by Hulett's (20) method and filtered between distillations. It was then sprayed repeatedly through a fine mesh cloth into a column of dilute nitric acid, washed with water in the same manner, and dried. It was then distilled twice more from a Hulett still and finally distilled *in vacuo*. The original mercury was part of a stock which had been repeatedly distilled for use in vacuum pumps.

The calomel was prepared as follows: Mercurous nitrate was formed by the action of redistilled nitric acid upon a large excess of the purified mercury. The product was thrown with the excess mercury into about 5 liters of "conductivity water" and calomel precipitated by the addition of dilute redistilled hydrochloric acid. Calomel cells prepared previously from calomel which had been washed with suction had not agreed as well as was expected, so the calomel, in this instance, was washed entirely by decantation in order to avoid change in grain size. Since washing by decantation is a very inefficient process it was continued for several days—in the preparation of 0.1 N calomel electrodes for several weeks—with renewals of 0.1 N KCl solution.

The KCl solution used for the calomel electrodes was prepared from Kahlbaum's *zur Analyse KCl*. The salt was recrystallized three times from "conductivity water" of specific conductivity of about  $1 \times 10^{-6}$  reciprocal ohms, ignited, and weighed with standardized weights with correction for air displacement. The solution was made up with "conductivity water" saturated with calomel containing a little mercury, and preserved in the dark in Jena bottles.

The 0.1 N calomel electrode vessels were made entirely of glass with no rubber stoppers.

*Weston Cells.*—Two Weston standard cells loaned by the Bureau of Standards were used. We are indebted to Mr. Shoemaker for the standardization of these cells. They were frequently used to check the commercial Weston cell which served as the working standard.

*Electrodes.*—Experience and the comments of others have taught us the advantage of freshly blacked electrodes when working with protein solutions. We have, therefore, adopted the use of palladium black instead of platinum black because the palladium can be dissolved off by electrolysis and there is no need to spoil the surface of the electrode with the heroic blast lamp treatment sometimes used. This was the sole reason for using palladium instead of platinum black in this investigation. The palladium black was deposited in some instances upon gold, in others upon platinum. We have found no difficulty in preparing electrodes which when thoroughly saturated with hydrogen and placed in the same solution agree within 0.02 millivolt or less. Their agreement in one solution, however, has seemed to us to be no certain guarantee of their agreement in another. Therefore, they were not compared apart from the main measurements but were assumed to agree when measurements of the same solution made with two electrodes in different vessels agreed.

We have made no search for the best means of depositing palladium black but have had good success by electrolysis, with a four volt storage battery, of a 3 per cent solution of palladium chloride containing a mere trace of lead acetate. The deposition should be carried far enough to produce a distinctly black coat but not so far that a thick layer is formed. No electrode was ever used unless the hydrogen liberated during the electrolysis in dilute sulfuric acid came off in perfectly even, small, and uniformly distributed bubbles. It was required that the deposit should be adherent enough to withstand a thorough washing with a vigorous jet of water.

*Hydrogen Electrode Vessels.*—These were of the type described in a previous paper (8). It may be reiterated here that the two chief principles which have been applied are: (1) the thorough establishment of the equilibria between hydrogen, electrode, and solution by shaking; and (2) the establishment of these equilibria within the vessel before the liquid contact is made so that the initial potential of the chain, before drifts of liquid diffusion potential set in, is significant. The first principle is Hasselbalch's (17); the second was suggested by the observations of Lewis and Rupert (22), Cumming and Gilchrist (12), and others on drifts of diffusion potentials. It should also be emphasized that the



electrode was fully immersed during a measurement but entirely exposed alternately to hydrogen and solution during the shaking.

*Hydrogen.*—Hydrogen was generated electrolytically from a 10 per cent NaOH solution, and was liberated from a nickel electrode. It was passed over sulfuric acid to take up the spray and then over heated platinized asbestos to burn out residual traces of oxygen. The generator was always kept in operation, on a low current over night, to avoid back diffusion of oxygen.

*Barometer Correction.*—Barometer readings were made with an instrument corrected by the Bureau of Standards. The readings were corrected for temperature, instrumental error, scale, and latitude. The pressure of hydrogen was considered to be that of the corrected barometer less the vapor tension of the solution as of pure water. The difference of pressure between that at the vessel and that at the hydrogen escapement was neglected since this escapement was through a free passage constricted for only 6 inches to about 3 mm. At times the influence of this constriction was made evident by back pressure in the hydrogen generator but it seldom if ever produced an excess pressure of over 0.2 mm. Hg.

Hydrogen electrode potentials were corrected to the *standard concentration* of hydrogen, which, contrary to the generally accepted standard in hydrogen electrode work, we have considered to be the concentration which the gas would have at 0°C. and 760 mm. corrected pressure.

*Temperature Control.*—An air bath was used for the maintenance of constant temperature. It consists of a well made cork-insulated box with an interior box placed so that there is an air chamber between it and the outer box on all sides but the front. A Sirocco centrifugal fan placed at one end draws air through this interior box and sends it back through the air chambers to the other end. In this way the air stream is kept free from serious eddy currents. The fan is belt-driven from the exterior.

Manipulations are made through two hand-holes with sliding doors. Light is furnished through a double window. The radiant energy of the electric bulb is cut down by a screen of nickel sulfate solution.

The heating coil is of No. 30 bare nichrome wire strung between asbestos board supports about the fan where it receives the most

vigorous air current. The electric current passing through this coil is adjusted in accordance with the room temperature so that the rate of heating when the current is thrown on by the regulator is approximately equal to the rate at which the air cools when the heat is off.

The thermoregulator is of the very sensitive type described in a former note (7)

The temperature maintained during these experiments was  $20^{\circ}\text{C}$ . according to the indications of a well seasoned thermometer calibrated by the Bureau of Standards and certified to be accurate to  $\pm 0.02^{\circ}\text{C}$ . Richards' point was taken by means of carefully recrystallized sodium sulfate and the thermometer found to be correct at that point within the above limit. Smaller fluctuations were estimated by means of a tapped Beckmann thermometer. Previous experiments with a thermo element had shown the temperature to vary  $\pm 0.003^{\circ}$  as the heat went on or off. This variation was about a very slowly drifting mean. Because of the great variation in the temperature of the room,  $11-18^{\circ}\text{C}$ ., the drifts were somewhat larger during the period of these experiments than in previous work at  $30^{\circ}$ , but they seldom exceeded  $\pm 0.05^{\circ}$  at any time; in the great majority of cases they were very much less, and during measurements were not over  $\pm 0.03^{\circ}$ .

All solutions were kept in the bath at least 2 hours before measurement. They were held in Jena glass Erlenmeyer flasks covered tightly with tinfoil.

*Potentiometer Equipment.*—Potential measurements were made with a Leeds and Northrup potentiometer calibrated by the Bureau of Standards. The rubber plate on which the coils and switches are mounted has been kept clean and free from the effect of light. The slide wire is frequently cleaned by gentle rubbing with a soft vaselined cloth. The galvanometer is a Leeds and Northrup type HN instrument. It is mounted against a solid brick wall with rubber supports. The telescope support was removed from the galvanometer case and remounted out of contact with the galvanometer. With these precautions all serious tremors were avoided. All wire connections on the switch board are soldered and the copper switches kept dusted.

Wires are heavily insulated and the system is shielded. No significant thermoelectromotive forces or leaks have been detected.

*The Solutions.*

In the preparation of all solutions volumetric flasks with volume at 20°C. certified by the Bureau of Standards were used. These flasks were, of course, treated with care and were never heated or undercooled. Standardized weights were used in weighing solid substances, but, with the exception of the KCl for the calomel electrodes, corrections for displacement of air were not made.

The water used in the preparation of all solutions had a specific conductivity of about  $1 \times 10^{-6}$  reciprocal ohms.

*Acid Potassium Phthalate.*—This salt was made by the method of Dodge (14) from sublimed phthalic anhydride and c.p. KOH. It was recrystallized twice from distilled water and thrice more from "conductivity water" and dried at 110–115° in platinum for 2 days. 0.2 M solutions were made by dissolving 40.828 gm. in "conductivity water" and making the solution up to 1 liter at 20°C.

*Acid Potassium Phosphate.*—A c.p. grade of this salt was recrystallized several times from distilled and from "conductivity water." It was dried 2 days in platinum at 110–115°C. 0.2 M solutions were made by dissolving 27.231 gm. in "conductivity water" and making up to a liter at 20°C.

*Boric Acid.*—This was recrystallized several times, dried between filter paper during 2 dry winter days, and the constancy of weight determined with small samples held for 2 weeks in a desiccator over  $\text{CaCl}_2$ . 0.2 M solutions were prepared by dissolving 12.4048 gm. in "conductivity water" and making up to 1 liter at 20°.

*Potassium Chloride.*—Kahlbaum's *zur Analyse KCl* was recrystallized twice from "conductivity water" and ignited in platinum. The saturated solution used to reduce contact potentials was not made from the recrystallized salt but from Kahlbaum's c.p.

*Boric Acid-Potassium Chloride Solution.*—This solution contained 12.4048 gm.  $\text{H}_3\text{BO}_3$  and 14.912 gm. KCl in 1 liter.

*Sodium Hydroxide.*—100 gm. of a high grade "NaOH from Na" were dissolved in 100 cc. of water. The Jena glass vessel was stoppered and allowed to stand about 18 hours until the precipitated carbonate had mostly settled. Instead of letting it stand longer in the glass and pipetting off the clear hydroxide solution as

Sörensen (36) recommends, it was filtered with suction through a paper which had been treated as follows: A hardened filter paper was placed for half an hour in warm 1 : 1 sodium hydroxide solution. The alkali solution was decanted and the paper washed first with absolute alcohol and then with more dilute alcohol, and finally with suction by copious quantities of distilled water. The paper, now on a small Buchner funnel under gentle suction, was allowed to dry partially but not to such an extent that it began to curl. The concentrated sodium hydroxide solution was then poured upon the middle, spread with a glass rod, and gently sucked through. The resulting filtered solution appeared perfectly free from suspended carbonate. No special precautions were used to prevent access of  $\text{CO}_2$  while diluting this solution except that exposure was made as short in duration as possible. It was diluted first to about a 2 N solution in a stoppered bottle. This was standardized roughly and the solution was then diluted to approximately 0.2 N. This was then poured quickly into a *paraffined* bottle and guarded with soda lime tubes. The burette was permanently connected with this bottle but without rubber connections.

The resulting 0.2 N solution contained so little carbonate that 50 cc. when run into a boiled barium chloride<sup>3</sup> solution in a flask protected by a Bunsen valve gave only a very slight cloudiness which could sometimes be seen only by using a ray of sunlight. If the flask was then opened to the laboratory air, a marked cloudiness developed in a few minutes.

This method of preparing "carbonate-free" sodium hydroxide solution is simple enough to be used in any laboratory, and it furnishes solutions which are doubtless pure enough for the present purpose.

<sup>2</sup> Mr. T. C. Trescot of the Bureau of Chemistry has for several years been using paper to filter concentrated sodium hydroxide solutions required in the determination of nitrogen by the Kjeldahl method. Dilute alkali solutions will, of course, disintegrate untreated paper; concentrated solutions will doubtless dissolve a small proportion of the paper treated as described, but there is no reason to believe that the organic matter carried into the solution during the time of filtration will be appreciable.

<sup>3</sup> This test is not of extreme delicacy but serves to show that sodium hydroxide solutions prepared as described are relatively free from carbonate.

*Hydrochloric Acid.*—A redistilled 20 per cent solution was diluted approximately to 0.2 N with "conductivity water."

*Standardization of Solutions.*—The phthalate, phosphate, and boric acid-potassium chloride solutions were standardized by weight.

The sodium hydroxide solution was standardized by titrating weighed quantities of acid potassium phthalate in a current of  $\text{CO}_2$  free air, using phenolphthalein as the indicator.

The hydrochloric acid was standardized by weighing as silver chloride.

The hydrochloric acid and sodium hydroxide solutions were then compared by titrating the acid with the alkali in a current of  $\text{CO}_2$  free air using phenolsulfonephthalein as the indicator.

In this way the normality of the sodium hydroxide solution was found to be:

By phthalate.....	0.18597
By HCl (AgCl) .....	0.18589

The proper factors for the hydrochloric acid and sodium hydroxide solutions were used so that results could be stated in terms of exact 0.2 N solution.

*Preparation of Mixtures.*—The acid potassium phthalate, acid potassium phosphate, and boric acid-KCl solutions were measured from clean calibrated pipettes into a 200 cc. flask. The 0.2 N HCl or 0.2 N NaOH, as the case might be, was then delivered from a calibrated burette, the mixture diluted to 200 cc. with "conductivity water," poured into a Jena Erlenmeyer flask, capped tightly with tinfoil, and placed in the air bath at least 2 hours before measurement. Since the ratio of acid to base is important, care was taken that one solution was not appreciably warmer than the other when measured; but since very slight dilutions of the mixture have no appreciable effect upon the hydrogen ion concentration, no particular care was taken to bring the solution exactly to  $20^\circ\text{C}$ . on dilution.

#### *Accuracy of Measurements and Values Involved.*

The potentials are given to the fifth decimal place because in most cases the observed potentials agreed within about 0.02–0.03 millivolt of the mean. In the subsequent calculation of the

hydrogen electrode potentials and  $P_{\pi}$  values, there are involved the theoretical potential of the 0.1 N calomel electrode referred to the normal hydrogen electrode as the standard or null point, the deviation therefrom of the particular calomel electrodes used in this investigation, and the correction to be applied for diffusion potentials. Let us first consider diffusion potentials.

*Uncertainties of Diffusion Potentials.*—In the older work and much of the later work, the corrections for diffusion potentials have been deduced from theoretical considerations embodied in equations such as those of Planck (34), Henderson (18), Cumming (10, 11), and Lewis and Sargent (21). Bjerrum (4, 5), however, developed the use of an interposed saturated solution of potassium chloride, and showed, not only that the diffusion potential is reduced, but that a correction may be applied by an extrapolation estimated from the potentials observed when the interposed solution is in one case saturated and in the next case half-saturated potassium chloride solution.

It should be remembered, however, that Bjerrum's method does not correct exactly, and Bjerrum himself called attention also to variations in diffusion potential, variations whose observation by Chanot (6) apparently led Henderson (18) to revise the formula for diffusion potential. These variations or drifts have been reported by Weyl (39), Bjerrum (4), Lewis and Rupert (22), Cumming and Gilchrist (12), Walpole (38), and others.

Bjerrum concluded that the time change should be inversely proportional to the thickness of the mixed layer between adjacent solutions. Cumming and Gilchrist (12) found their own experiments to be "in general accord with Bjerrum's even though there are still some points of difficulty." They concluded, however, that the values deduced by means of one or another formula are not realized experimentally.

Our own somewhat limited observations on diffusion potential have brought us into entire agreement with the suggestions of Cumming and Gilchrist, which are as follows:

1. To obtain accurate measurements of potential it is necessary to form a new boundary shortly before the measurement is taken; otherwise a serious error may be introduced.

2. It is desirable that the two solutions should be mechanically mixed at the boundary and this is the more important the narrower the tube in which the boundary is formed.

3. Capillary tubes should be altogether avoided in the construction of an electromotive cell.

4. The electromotive force of any cell which derives part of its potential from the presence of a liquid contact is not constant but varies with the time which has elapsed since formation of the boundary; if due attention is paid to the precautions mentioned above, constant and comparable results may be obtained."

Myers and Acree (32) seem to have arrived at a different point of view. They were doubtless aware of the fact that the drift in diffusion potential had been observed by others with systems in which the only variation which occurred was shown to be the potential at the liquid contact. Myers and Acree, however, state:

"Several investigators, and especially Bjerrum, have observed that in measuring any system in which the hydrogen electrode is a part there is a change in the value obtained at the beginning and an hour later, perhaps. . . . If the systems  $0.1\text{ N KCl-HgCl-Hg}$  and  $0.1\text{ N HCl-Pt-H}_2$  are to be compared, it is possible to connect them by satisfactory means and still obtain a changing value for the system if it is not first saturated with hydrogen. But suppose that the connecting system<sup>4</sup> is first saturated with hydrogen, or, in other words, equilibrium is established and the systems are connected. Observations over periods of even a couple of hours show no changes whatever, provided no barometric change has taken place."

It may be significant to note that Cumming and Gilchrist have given a theoretical reason for believing that the time change should be small for the particular ratio of KCl to HCl mentioned in the above quotation, and that the time change would be small if the liquid contact were made in a large tube.

With the above quotation in mind, but with an entirely different purpose in view, the hydrogen electrode vessel described in a previous paper (8) was designed so that the equilibria within the vessel could be established before the liquid contact was made. In spite of this, we have found it impossible to obtain constant and at the same time reproducible potentials with hydrochloric acid solutions when a saturated solution of potassium chloride was used as the connecting fluid. A fair degree of repro-

<sup>4</sup> Dr. Myers tells us that by saturation of the "connecting system" with hydrogen the interposed potassium chloride solution was not meant, but only the solution whose hydrogen ion concentration was to be measured. A portion of this becomes part of the "connecting system."

ducibility could be obtained with hydrochloric acid solutions if the potential was measured directly after the formation of the liquid contact. This has been found to hold true for all the solutions tested. After this first reading a drift of potential has always been observed. The magnitude of this drift is comparatively insignificant in most cases, but with hydrochloric acid it is distinct and serious. In general the rate of drift is proportional to the magnitude of the diffusion potential estimated by the Bjerrum extrapolation.

It might be urged that this large drift with hydrochloric acid solutions was due to contamination of the solution at the electrode by the diffusion into the vessel of potassium chloride. This will not explain why potentials showed less change with more dilute hydrochloric acid solutions (the difference in density between 0.01  $N$  HCl and 0.002  $N$  HCl could play little part in 5 minutes), or why only negligible changes were observed with other solutions, or the very good parallelism between the magnitude of this drift and the drift observed when the contact was formed between constant calomel electrodes.

Experimental work like that of Cumming and Gilchrist is invaluable, for the biochemist must perforce study solutions whose diffusion potentials cannot be calculated. He must therefore depend upon the experimental reduction of diffusion potentials by some means such as the use of an interposed saturated solution of potassium chloride, and he can gain little help in the numerous papers on the calculation of diffusion potentials unless these lead, as did Bjerrum's work, to a basis for further correction. The Bjerrum extrapolation, however, was worked out with hydrochloric acid solutions and we know of no good reason why it should be considered applicable to all sorts of solutions except as a probable index of the magnitude of the uneliminated diffusion potential.

Nevertheless, if the magnitude of the Bjerrum extrapolation and the rate of drift in diffusion potentials are good criterions, many of the solutions used by the biochemist, whether buffer mixtures or biological fluids, have much smaller diffusion potentials against a saturated solution of potassium chloride than hydrochloric acid solutions.



The considerations stated above point to the conclusion that hydrochloric acid solutions are not well suited to routine hydrogen electrode standardization of calomel electrodes—a point to which we shall return later. Such solutions, however, have been used almost exclusively in determining the theoretical potential of the 0.1 N or normal calomel electrode in terms of the normal hydrogen electrode. Let us consider briefly some of the values given in the literature, touching first upon the definition of the “normal hydrogen electrode.”

*The Normal Hydrogen Electrode.*—The normal hydrogen electrode is commonly supposed to be a platinized electrode immersed in a solution normal with respect to hydrogen ions and under an atmosphere of pure hydrogen and water vapor with the hydrogen at a partial pressure of 760 mm. One of us (9) will suggest in a paper now in manuscript that the definition should be revised to specify that the hydrogen gas should be at the *standard concentration* which is the concentration which the gas would have if it were under 760 mm. pressure and at 0°C.

This additional correction for the temperature of the hydrogen, like the correction for displacement of hydrogen by the vapor of a solution, is of no importance in standardizing the calomel electrode by means of a hydrogen electrode, provided the values used in any investigation all exclude or all include the correction and are all derived from data established at the temperature under consideration. But in comparing data derived at different temperatures it should be noted that the correction for both the displacement of hydrogen by water vapor and the rarefaction of the hydrogen due to its temperature is more than 0.8 millivolt greater at 30° than at 18°, and that at 30° and 760 mm. the total correction for vapor tension and temperature amounts to 1.92 millivolts.

With this in mind let us then compare some values assigned to the 0.1 N calomel electrode at different temperatures.

*Values Assigned to the 0.1 N Calomel Electrode.*—In Table I will be found some values referred to the normal hydrogen electrode under 760 mm. pressure of “wet” hydrogen. These have been corrected first to the normal hydrogen electrode under a partial pressure of 760 mm. hydrogen and then to the normal density or concentration of hydrogen at 0°C. and 760 mm. pressure.

TABLE I.

Potentials of the 0.1 N KCl calomel electrode referred to the normal hydrogen electrode at different temperatures and different standards of hydrogen concentration.\*

Author.	Temperature.	Potential referred to normal hydrogen electrode at indicated concentration of hydrogen. 760 mm.		
		"Wet" at $t^{\circ}$ .	"Dry" at $t^{\circ}$ .	"Dry" at $0^{\circ}$ .
	$^{\circ}\text{C.}$	volts	volts	volts
Bjerrum.....	0	0.3366	0.3367	0.3367
Sørensen and Koefoed.....	18	0.3377	0.3380	0.3388
" " ".....	20	0.3375	0.3378	0.3387
Clark.....	20	0.3373	0.3376	0.3385
Bjerrum.....	25	0.3367	0.3371	0.3382
Sørensen and Koefoed.....	30	0.3364	0.3370	0.3383
" " ".....	40	0.3349	0.3359	0.3378
" " ".....	50	0.3326	0.3344	0.3368
" " ".....	60	0.3290	0.3321	0.3350
Bjerrum.....	75	0.3243	0.3315	0.3351

It is worthy of notice that the difference of 1.3 millivolts between Sørensen and Koefoed's (35) values at 18 and  $30^{\circ}$  reduces to 0.5 millivolt when fully corrected, and that the difference of 12.3 millivolts between Bjerrum's measurements  $75^{\circ}$  apart reduces to 1.6 millivolts when the full correction is applied.

All our measurements have been corrected to 760 mm. partial pressure of hydrogen at  $0^{\circ}$ . In a sense, it may seem superfluous to make all these corrections for biochemical purposes, but the correction for changing barometer is necessary, and, when once the barometric reading has received the proper corrections, all the others can be found from a plotted curve in one operation.

For the convenience of others, we have tabulated in Table II the full corrections to be applied at different temperatures and barometric readings. These can be plotted so that the application of the correction is easy.

We have included in Table I only the measurements of Bjerrum and of Sørensen and Koefoed because they appear to be comparable enough to use in the foregoing discussion.

Loomis and Acree (27) have presented a choice of values at  $25^{\circ}$  for the 0.1 N calomel electrode which depend upon the per-

the full correction for the hydrogen pressure. This happens to be identical with the average of Bjerrum's and Sørensen's corrected values at 18–30° (Table I), but the maximum variation from the mean was 0.22 millivolt and the mean variation 0.14, and the diffusion potential had to be averaged from a large number of measurements, some of which differed by 0.4 millivolt. The details of these measurements are hardly worth publishing since no very illuminative order was found in the conduct of the diffusion potentials.

The value 0.3385, referred to a normal hydrogen electrode under standard concentration of hydrogen gas (760 mm. at 0°C.), is the value we have assigned to the average of our 0.1 N calomel electrodes. It is a somewhat arbitrary value based on the considerations presented above and upon the older values for the dissociation of hydrochloric acid solutions. If it differs radically from Lewis and Randall's value and from the values given by Loomis and Acree, it is, at least, a value in harmony with those generally accepted in biochemical work, and places our data on the "buffer mixtures" in harmony with a scale of hydrogen ion concentration whose importance has become too great to justify hasty resetting.

It should be emphasized again that it is to a large extent an arbitrary value which will have to be revised when the true value shall have been so well established that there will be no hesitancy in shifting the scale used in biochemistry.

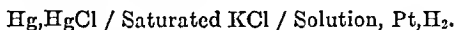
In addition to the unsatisfactory state of the question of the theoretical value to be assigned to the 0.1 N calomel electrode, we find a vexatious uncertainty in the potentials of our particular individual calomel electrodes. In the pressure of other researches we have discarded some sixty electrodes because they drifted apart sometimes as much as half a millivolt. In this investigation one of the six electrodes persisted in a potential 0.2 to 0.3 millivolt higher than the others. These other five differed among themselves 0.02 to 0.08 millivolt. We have been persistently defeated in the effort to prepare calomel electrodes agreeing better than the six used in the present investigation, and we are inclined to believe that we can improve the agreement only by adopting the Lipseomb and Hulett (25) method of preparing the calomel electrolytically. In view of Ellis' (15) excellent results with this method applied to HCl calomel electrodes, it is hardly

worth while to state more than the above summary of the disagreement of our electrodes since this is sufficient to indicate our order of accuracy. The device used by Myers and Acree (32) which consists in short circuiting differences in potentials was not resorted to.

### *The Measurements.*

The measurements are given in Tables III to VI.

At the head of each table will be found the composition of the mixtures. In the first column are given the cc. of acid or alkali added as indicated in the heading. Then follows the average observed potential of the chain:



This potential is next corrected to the standard concentration of hydrogen at 0° and 760 mm. pressure with the assumption that the vapor pressure of the solution is that of pure water at the temperature used. The potential is then corrected to the basis of the average of the six 0.1 N calomel electrodes, and finally it is corrected for the diffusion potential by Bjerrum's extrapolation. The next to the last column contains the corrected hydrogen electrode potential found by subtracting the assumed standard potential of the 0.1 N calomel electrode, and finally in the last column is given the calculated value of Sørensen's  $P_H$  or  $-\log [C_H]$ .

In this calculation the familiar equation was used:

$$\Pi = \frac{RT}{nF} \ln \frac{1}{[C_H]}$$

R = Gas constant in volt coulombs (8.31574).

T = Absolute temperature,  $273.09 + t^\circ$ .

n = Valence of hydrogen, 1.

F = The Faraday, 96,500 coulombs.

ln = Natural logarithm. Factor for transposing to Briggsian logarithm, 0.4343.

At 20°C. the above equation becomes:

$$\Pi = 0.058155 \log_{10} \frac{1}{[C_H]} = 0.058155 P_H$$

TABLE III.

Hydrogen electrode measurements of *phthalate-HCl* mixtures at 20° C.  
Composition of solutions: 50 cc. 0.2 M  $\text{KHC}_8\text{H}_4\text{O}_4$  + X cc. 0.2 N HCl.  
Diluted to 200 cc.

X	E. M. F. observed.	E. M. F. corrected for barometer.	E. M. F. corrected to average 0.1 N calomel electrode.	E. M. F. corrected for diffusion potential.	E. M. F. corrected - 0.3355.	$P_H$
	volts	volts	volts	volts	volts	
0	0.47995	0.48154	0.56976	0.56938	0.23088	3.970
1	0.47620	0.47779	0.56601	0.56564	0.22714	3.906
5	0.46202	0.46319	0.55139	0.55104	0.21254	3.655
10	0.44697	0.44814	0.53634	0.53602	0.19752	3.396
15	0.43499	0.43616	0.52436	0.52405	0.18555	3.191
20	0.42450	0.42568	0.51388	0.51358	0.17508	3.011
25	0.41496	0.41614	0.50434	0.50405	0.16555	2.847
30	0.40590	0.40708	0.49528	0.49501	0.15651	2.691
35	0.39698	0.39816	0.48636	0.48611	0.14761	2.533
40	0.38830	0.38948	0.47768	0.47745	0.13895	2.389
45	0.37992	0.38110	0.46930	0.46909	0.13059	2.246
50	0.37226	0.37344	0.46164	0.46144	0.12294	2.114

TABLE IV.

Hydrogen electrode measurements of *phthalate-NaOH* mixtures at 20° C.  
Composition of solutions: 50 cc. 0.2 M  $\text{KHC}_8\text{H}_4\text{O}_4$  + X cc. 0.2 N NaOH.  
Diluted to 220 cc.

X	E. M. F. observed.	E. M. F. corrected for barometer.	E. M. F. corrected to average 0.1 N calomel electrode.	E. M. F. corrected for diffusion potential.	E. M. F. corrected - 0.3355.	$P_H$
	volts	volts	volts	volts	volts	
0	0.48051	0.48156	0.56977	0.56939	0.23089	3.970
1	0.48421	0.48526	0.57347	0.57308	0.23458	4.034
2	0.48790	0.48897	0.57718	0.57678	0.23828	4.097
5	0.49806	0.49913	0.58734	0.58692	0.24842	4.272
10	0.51207	0.51316	0.60137	0.60093	0.26243	4.513
15	0.52330	0.52439	0.61260	0.61214	0.27364	4.705
20	0.53308	0.53435	0.62253	0.62206	0.28356	4.876
25	0.54242	0.54369	0.63187	0.63139	0.29289	5.036
30	0.55205	0.55333	0.64151	0.64103	0.30253	5.202
35	0.56260	0.56388	0.65206	0.65158	0.31308	5.353
40	0.57563	0.57691	0.66509	0.66461	0.32611	5.608
45	0.59583	0.59711	0.68529	0.68480	0.34630	5.955
47	0.61009	0.61137	0.69955	0.69905	0.36055	6.200
48	0.62099	0.62229	0.71047	0.70996	0.37146	6.357
49	0.63982	0.64112	0.72930	0.72878	0.39028	6.711
50	(0.74010)	0.74140	0.82958	0.82905	0.49055	(8.435)

TABLE V.

Hydrogen electrode measurements of "phosphate mixtures" at 20° C. Composition of solutions: 50 cc. 0.2 M  $\text{KH}_2\text{PO}_4$  + X cc. 0.2 N NaOH. Diluted to 200 cc.

X	E. M. F. observed.	E. M. F. corrected for barometer.	E. M. F. corrected to average 0.1 N calomel electrode	E. M. F. corrected for diffusion potential.	E. M. F. corrected - 0.3385.	P <sub>H</sub>
	volts	volts	volts	volts	volts	
1	0.55346	0.55468	0.64293	0.64267	0.30417	5.230
2	0.57076	0.57200	0.66025	0.65997	0.32147	5.528
5	0.59449	0.59573	0.68398	0.68366	0.34516	5.935
10	0.61423	0.61547	0.70372	0.70337	0.36487	6.274
20	0.63770	0.63894	0.72719	0.72680	0.38830	6.677
25	0.64757	0.64871	0.73686	0.73646	0.39796	6.843
30	0.65745	0.65859	0.74674	0.74633	0.40783	7.013
40	0.68142	0.68266	0.77081	0.77039	0.43189	7.427
45	0.70187	0.70314	0.79129	0.79084	0.45234	7.778
47	0.71606	0.71733	0.80548	0.80500	0.46650	8.022

TABLE VI.

Hydrogen electrode measurements of "borate mixtures" at 20° C. Composition of solutions: 50 cc. 0.2 M  $\text{H}_3\text{BO}_3$ , 0.2 M KCl + X cc. 0.2 N NaOH. Diluted to 200 cc.

X	E. M. F. observed.	E. M. F. corrected for barometer	E. M. F. corrected to average 0.1 N calomel electrode.	E. M. F. corrected for diffusion potential	E. M. F. corrected - 0.3385	P <sub>H</sub>
	volts	volts	volts	volts	volts	
0	(0.57400)	0.57511	0.66343	0.66343	0.32493	(5.587)
1	0.67680	0.67791	0.76623	0.76623	0.42773	7.355
2	0.69521	0.69629	0.78459	0.78459	0.44609	7.671
3	0.70633	0.70743	0.79573	0.79573	0.45723	7.862
4	0.71447	0.71557	0.80386	0.80386	0.46536	8.002
5	0.72108	0.72218	0.81047	0.81046	0.47196	8.116
10	0.74258	0.74391	0.83232	0.83230	0.49380	8.491
15	0.75720	0.75853	0.84694	0.84691	0.50841	8.742
25	0.78023	0.78156	0.86997	0.86991	0.53141	9.138
35	0.80243	0.80376	0.89217	0.89209	0.55359	9.519
40	0.81609	0.81742	0.90583	0.90573	0.56723	9.754
45	0.83566	0.83699	0.92540	0.92529	0.58679	10.090
47	0.84763	0.84896	0.93737	0.93725	0.59875	10.296
50	0.87820	0.87953	0.96794	0.96782	0.62932	10.821

TABLE VII.

$P_H$  values of *KCl-HCl mixtures*. Preliminary measurements at 25°C. Composition of solutions: 50 cc. 0.2 M KCl + X cc. 0.2 N HCl. Diluted to 200 cc.

X	$P_H$	X	$P_H$
2	2.733	20	1.722
5	2.332	25	1.625
7	2.180	40	1.416
10	2.027	50	1.314
15	1.847		

## DISCUSSION.

The observed potentials given in the tables are the average of at least two measurements agreeing within  $\pm 0.03$  millivolt of the mean. The onset of warm weather has prevented repetition of these measurements at 20° with the present equipment, but they will be repeated at higher temperatures in order to establish the temperature coefficients.

0.05 M instead of the higher concentrations have been studied for two reasons. In the first place, solutions as concentrated as 0.1 M are at the border line of what may be considered the range of dilute solutions and are therefore less adapted to the theoretical treatment which may become necessary. In the second place, we have desired for our own work a series of solutions whose salt concentration is more nearly comparable with that of ordinary bacteriological culture media than are the solutions generally used.

The addition of potassium chloride to the borate mixtures was found advisable in order to make the "salt effect" upon indicators comparable in the borate and phosphate mixtures. There is a certain inconsistency in retaining the same concentration of potassium chloride as the salt in the form of borate increases. This might be overcome by dilution of the borate mixtures with declining volumes of a potassium chloride solution, but we have considered this unwise in view of the fact that careless errors in the volume of potassium chloride might introduce a larger effect upon the hydrogen ion concentration of the solution than the "salt error" of the present method. Furthermore, the more alka-

line borate mixtures where this salt error might become appreciable will seldom need to be used in bacteriological or physiological investigations. The effect of the potassium chloride upon the  $P_H$  values of borate mixtures was shown by a rise of about  $0.04 P_H$ . Similar effects of neutral salts have been discussed by Sørensen (36), Loomis and Acree (27), Walpole (38), and Harned (40).

Since potential measurements of "unbuffered" mixtures cannot be accurately made, the potentials observed with such mixtures are bracketed.

For bacteriological colorimetric work we have found it convenient to use these mixtures at intervals of  $0.2 P_H$ . Interpolation to  $0.1$  is easy in most cases and the accuracy allowed is adequate for all general work. For the convenience of those who wish to use this system we have given in Table VIII the compositions corresponding to intervals of  $0.2 P_H$ . We make up the full 200 cc. of solution instead of the 10 cc. of other systems because one generally wishes several portions of a solution in a day's work, and because, by preserving these solutions in properly stoppered bottles each with its own 10 cc. pipette, there is always some at hand for the occasional test. All solutions should be kept in resistant glass and the borate mixtures in paraffined bottles.

It will be found that the more acid phthalate-hydrochloric acid solutions contain so much free phthalic acid that it will crystallize out at  $20^\circ$ . The separation of the phthalic acid probably produces an insignificant change in the hydrogen ion con-

TABLE VIII.

Composition of mixtures giving  $P_H$  values at  $20^\circ\text{C}$ . at intervals of  $0.2 P_H$ .

$P_H$	Phthalate-HCl mixtures.			
2.2	50 cc. 0.2 M KH Phthalate	46.70 cc. 0.2 N HCl.	Diluted to 200 cc.	
2.4	50 " 0.2 " "	39.60 " 0.2 " "	" " "	"
2.6	50 " 0.2 " "	32.95 " 0.2 " "	" " "	"
2.8	50 " 0.2 " "	26.42 " 0.2 " "	" " "	"
3.0	50 " 0.2 " "	20.32 " 0.2 " "	" " "	"
3.2	50 " 0.2 " "	14.70 " 0.2 " "	" " "	"
3.4	50 " 0.2 " "	9.90 " 0.2 " "	" " "	"
3.6	50 " 0.2 " "	5.97 " 0.2 " "	" " "	"
3.8	50 " 0.2 " "	2.63 " 0.2 " "	" " "	"



TABLE VIII—Concluded.

$P_H$	Phthalate—NaOH mixtures.				
4 0	50 cc. 0.2 M KH Phthalate	0.40 cc. 0.2 N NaOH.	Diluted to 200 cc.		
4 2	50 " 0.2 " "	3.70 " 0.2 " "	"	"	"
4.4	50 " 0.2 " "	7.50 " 0.2 " "	"	"	"
4.6	50 " 0.2 " "	12.15 " 0.2 " "	"	"	"
4.8	50 " 0.2 " "	17.70 " 0.2 " "	"	"	"
5.0	50 " 0.2 " "	23.85 " 0.2 " "	"	"	"
5 2	50 " 0.2 " "	29.95 " 0.2 " "	"	"	"
5 4	50 " 0.2 " "	35.45 " 0.2 " "	"	"	"
5 6	50 " 0.2 " "	39.85 " 0.2 " "	"	"	"
5 8	50 " 0.2 " "	43.00 " 0.2 " "	"	"	"
6 0	50 " 0.2 " "	45.45 " 0.2 " "	"	"	"
6 2	50 " 0.2 " "	47.00 " 0.2 " "	"	"	"
$P_H$	$KH_2PO_4$ —NaOH mixtures.				
5 8	50 cc. 0.2 M $KH_2PO_4$	3.72 cc. 0.2 N NaOH.	Diluted to 200 cc.		
6 0	50 " 0.2 " "	5.70 " 0.2 " "	"	"	"
6 2	50 " 0.2 " "	8.60 " 0.2 " "	"	"	"
6 4	50 " 0.2 " "	12.60 " 0.2 " "	"	"	"
6 6	50 " 0.2 " "	17.80 " 0.2 " "	"	"	"
6 8	50 " 0.2 " "	23.65 " 0.2 " "	"	"	"
7 0	50 " 0.2 " "	29.63 " 0.2 " "	"	"	"
7 2	50 " 0.2 " "	35.00 " 0.2 " "	"	"	"
7 4	50 " 0.2 " "	39.50 " 0.2 " "	"	"	"
7 6	50 " 0.2 " "	42.80 " 0.2 " "	"	"	"
7 8	50 " 0.2 " "	45.20 " 0.2 " "	"	"	"
8 0	50 " 0.2 " "	46.80 " 0.2 " "	"	"	"
$P_H$	Boric acid, KCl—NaOH mixtures.				
7 8	50 cc. 0.2 M $H_3BO_3$ , 0.2 M KCl	2.61 cc. 0.2 N NaOH.	Diluted to 200 cc.		
8 0	50 " 0.2 " "	3.97 " 0.2 " "	"	"	"
8 2	50 " 0.2 " "	5.90 " 0.2 " "	"	"	"
8 4	50 " 0.2 " "	8.50 " 0.2 " "	"	"	"
8 6	50 " 0.2 " "	12.00 " 0.2 " "	"	"	"
8 8	50 " 0.2 " "	16.30 " 0.2 " "	"	"	"
9 0	50 " 0.2 " "	21.30 " 0.2 " "	"	"	"
9.2	50 " 0.2 " "	26.70 " 0.2 " "	"	"	"
9.4	50 " 0.2 " "	32.00 " 0.2 " "	"	"	"
9.6	50 " 0.2 " "	36.85 " 0.2 " "	"	"	"
9.8	50 " 0.2 " "	40.80 " 0.2 " "	"	"	"
10.0	50 " 0.2 " "	43.90 " 0.2 " "	"	"	"

centration. We hope to report upon measurements at higher temperatures in the near future.

The defects of this system may be summarized as follows:

1. Inasmuch as the hydrogen ion concentration of a mixture is determined chiefly by the ratio of acid to base, the substitution of NaOH in place of the  $\text{Na}_2\text{HPO}_4$  used by Sørensen in the phosphate mixtures and of NaOH in place of  $\text{Na}_2\text{B}_4\text{O}_7$  used by Palitzsch in the borate mixtures may introduce serious errors in the more alkaline mixtures of each series, if the sodium hydroxide and acid potassium phosphate or the sodium hydroxide and boric acid (KCl) are not standardized to the correct ratio.

2. Of the more acid mixtures of the phthalate-hydrochloric acid series, those more acid than  $\text{P}_n = 2.6$  are saturated with free phthalic acid which will crystallize out at low temperatures.

3. The correction for the salt error of indicators by the addition of potassium chloride to the more acid borate mixtures in order to make these comparable with the phosphate mixtures is continued in the more alkaline mixtures where the salts of boric acid doubtless produce a salt effect.

The second and third criticisms will probably have little significance for the particular use for which this set of mixtures was designed. It should be emphasized that the variation in the salt concentration of the mixtures we describe is very much less than that of some of the Sørensen mixtures; for example, the citrate mixtures.

The first mentioned defect is one which applies not only to these mixtures but to Sørensen's citrate mixtures, borate mixtures, and alkaline glycoll mixtures. It is compensated for by the fact that the sodium hydroxide may be prepared in sufficient purity and standardized with sufficient accuracy by simple means if the procedures outlined are followed, and also by the fact that all the solid substances used are easily purified, free from water of crystallization, unaffected by atmospheric carbon dioxide, and invested with many of the properties which make possible accurate preparation of their solutions.<sup>6</sup>

<sup>6</sup> In making this statement we are not unmindful of the fact that because a substance crystallizes beautifully it is no guarantee that it can thereby be perfectly freed from impurities. We believe, however, that three crystallizations of the substances mentioned, if properly done, will furnish material of a purity adequate for the purpose. We recommend that under no circumstances unrecrystallized material should be used.

Colorimetric checks upon the consistency of any particular set of these mixtures may be made by comparing "5.8" and "6.2" phthalate with "5.8" and "6.2" phosphate, using dipropyl red (28) and dibromothymolsulfonephthalein (28) as the indicators, and by comparing "7.8" and "8.0" phosphate with "7.8" and "8.0" borate, using phenolsulfonephthalein (28) and  $\alpha$ -naphtholphthalein as the indicators. The ratio of the hydrochloric to sodium hydroxide is susceptible to accurate determination, and one of the substances used in these mixtures, namely, acid potassium phthalate, is eminently adapted to the standardization of the sodium hydroxide solution.

The system of mixtures here described is not so complete as the admirable Sørensen mixtures, but just as Palitzsch found it best to alter the borate mixtures to make their preparation possible on long ship voyages, so we believe the system here outlined will appeal to the peculiar needs of the bacteriologist if it does not find a more general acceptance.

#### *Acid Potassium Phthalate as a Standard.*

In routine hydrogen electrode measurements some solution with well defined hydrogen ion concentration is generally chosen to standardize the particular system used as well as to check the accuracy or stability of the particular calomel electrode used. Any one of the buffer mixtures whose hydrogen ion concentration is well defined may be so employed. Thus Sørensen uses a citric acid-sodium citrate mixture and Michaelis has recommended the "standard acetate mixture" which Walpole has carefully studied. Others have used hydrochloric acid solutions. The difficulties attending the use of hydrochloric acid have been mentioned by others, especially Michaelis, and have been touched upon in this paper. The "standard citrate" and "standard acetate" solutions must be constructed by adjustment of their components, and the standard acetate solution, though excellent in some respects, loses acetic acid easily.

Acid potassium phthalate, on the other hand, possesses a somewhat unique combination of properties which makes its solution an excellent standard. Examination of the "titration curve," of phthalic acid (Fig. 2) will show at a glance that the first and

second dissociation constants are so close that the second hydrogen begins to dissociate before the first is neutralized. In other words, when the mono alkali salt is formed, the solution still retains a marked "buffer effect." A solution, therefore, of the crystalline salt, unlike solutions of practically all the salts of

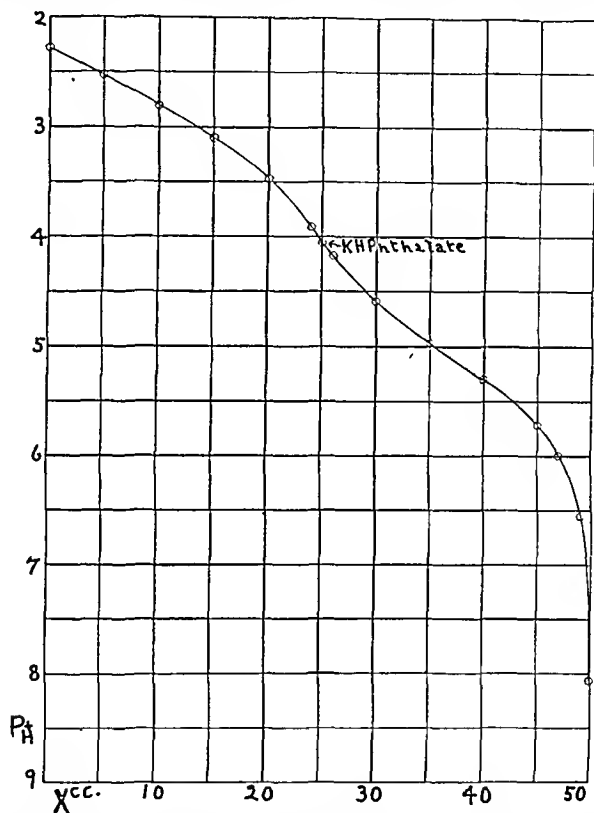


FIG. 2. Titration curve of *o*-phthalic acid at 30°. 150 cc. *m*/30 phthalic acid + X cc. 0.2 *M* NaOH. Diluted to 200 cc.

monobasic and the majority of polybasic acids, possesses a stable hydrogen ion concentration. This happens also to be sufficiently high to prevent atmospheric carbon dioxide from having much effect. When it is recalled that acid potassium phthalate can be prepared with high purity, can be easily recrystallized, and has

no water of crystallization, it would seem to be ideal for the standardization of a hydrogen electrode-calomel electrode system. Its apparent diffusion potential against saturated KCl, estimated by the Bjerrum extrapolation method, is somewhat higher than that of "standard acetate," but not seriously high. We have observed no sign of any reduction by the electrode such as occurs with sodium amalgam in very alkaline solutions. Unlike the acetic acid in "standard acetate" the phthalic acid is not volatile and therefore does not require the hydrogen to be bubbled through an elaborate train of the solution. Certainly for routine work it will be simpler to use a solution whose ratio of acid to base is determined by the crystals rather than by artful adjustment.

The comparison of phthalate solutions, "standard acetate," and other mixtures which are particularly suited to hydrogen electrode measurements can furnish us with reliable fixed points such as those used in thermometry. So long as relative values only are needed, these points would suffice to subdivide a scale which would be extremely useful. Such a scale might be made totally independent of the potential of any secondary standard such as the calomel electrode, it need have no concern with any conception regarding the percentage dissociation of hydrochloric acid, and it could, if necessary, justify itself without leaning at any point upon conductivity measurements.

On the other hand, the necessity for correlating the data of different experimental methods makes it advisable to fix the position of the scale by standardization with a solution which is experimentally susceptible to a fundamental evaluation of its hydrogen ion concentration by methods not involving hydrogen electrode potentials. In many respects hydrochloric acid solutions are well suited for this, and our scale of hydrogen ion concentrations will doubtless continue for some time to have its position fixed by measurements with hydrochloric acid.

When opportunity can be found, we hope to make a careful comparison of phthalate, hydrochloric acid, "standard acetate," and other standard solutions. Therefore, we are not presenting our value for the potential of a 0.05 M acid potassium phthalate solution at 20°, which will be found in Tables III and IV, as our final value. It is, however, probably accurate enough for all routine uses.

## SUMMARY.

The hydrogen electrode potentials of the following mixtures have been studied at 20°C.

Acid potassium phthalate—hydrochloric acid.		
“ “ “ —sodium hydroxide.		
“ “ phosphate— “ “		
Boric acid + KCl — “ “		
Boric acid — “ “		
At 25°C. potassium chloride — hydrochloric acid.		

In all cases the concentration of the first component was maintained at 0.05 M.

The evaluation of the 0.1 N KCl calomel electrode in terms of the “normal hydrogen electrode” is discussed.

It is suggested that a temperature correction should be included in the barometric correction of hydrogen electrode potentials. The application of this together with a consideration of several theoretical and experimental difficulties leads to the conclusion that in biochemical work the 0.1 N KCl calomel electrode should be given the provisional and somewhat arbitrary value 0.3385 between 18 and 30°C. when referred to the potential of the “normal hydrogen electrode.”

By applying the full barometric correction and the above value of the 0.1 N calomel electrode the  $P_H$  values of the various mixtures were calculated, and the compositions given which furnish mixtures differing by intervals of 0.2  $P_H$  for use as comparison solutions in the colorimetric determination of hydrogen ion concentrations.

It is shown that the system of “buffer” mixtures described is somewhat more simple to prepare and easier to standardize than the systems in common use.

Acid potassium phthalate solutions possess properties which make them comparable with or better than “standard acetate” and similar solutions for standardizing hydrogen electrode systems.

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# THE CONJUGATED SULFURIC ACID OF THE MUCIN OF PIG'S STOMACH (MUCOITIN · SULFURIC ACID).

FIRST PAPER.

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(Received for publication, June 1, 1916.)

It has been demonstrated in recent years that a conjugated sulfuric acid combined with protein is widely distributed in animal tissues and that it is present even in leukocytes. The question of the identity or non-identity of the conjugated sulfuric acids of different origin could not be answered so long as there existed no detailed knowledge of the structure of any one substance of this group.

The work of Levene and La Forge<sup>1</sup> has furnished considerable information regarding the structure of the acid derived from cartilage, the chondroitin sulfuric acid discovered by Morner<sup>2</sup> and first studied by Schmiedeberg.<sup>3</sup>

The analysis of the conjugated sulfuric acid derived from tendomucoid followed, and revealed the identity of this substance with the original chondroitin sulfuric acid. Hence chondromucoid and tendomucoid can differ only by the nature of their protein components, if they possess any point of difference at all.

Very little is known about the relationship of mucoids to true mucin. First mention of the presence of a conjugated sulfuric acid in the molecule of true mucin was made by Levene,<sup>4</sup> and in recent years much work on this subject was done in Hofmeister's laboratory. López-Suárez<sup>5</sup> has isolated a conjugated

<sup>1</sup> Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1914, xviii, 237.

<sup>2</sup> Morner, C. T., *Skand. Arch. Physiol.*, 1889, i, 210.

<sup>3</sup> Schmiedeberg, O., *Arch. exp. Path. u. Pharm.*, 1891, xxviii, 358.

<sup>4</sup> Levene, P. A., *Biochem. Z.*, 1909, xvi, 246.

<sup>5</sup> López-Suárez, J., *Biochem. Z.*, 1913, lvi, 167.



sulfuric acid from the mucin occurring in pig's stomach, and Alzona,<sup>6</sup> employing Levene's picric acid method, isolated the same substance from the mucous membrane of the stomach, the intestines, the prostate, the bladder, and from the thyroid.

The present work deals with the conjugated sulfuric acid obtained from the mucin occurring in the pig's stomach. The substance was prepared by the same process as described by Levene and La Forge for tendomucin.

In this phase the substance contained besides sulfuric also phosphoric acid. For further purification use was made of a process previously described by Levene; namely, the crude conjugated sulfuric acid was separated from the nucleic acids as a water-soluble barium salt. However, the nucleic acid was removed only after many repeated precipitations. The final product contained practically no phosphorus at all, but it apparently was partially hydrolyzed by the continued treatment with barium hydroxide. In its carbon:nitrogen ratio the substance came nearer to the original chondroitin sulfuric acid than the samples analyzed in Hofmeister's laboratory.

	Calculated for $C_{25}H_{40}N_2S O_{10}Ba_2$	Found for substances obtained by			
		Levene. <sup>1</sup>	Levene and La Forge. <sup>2</sup>	Levene and López-Suárez.	López-Suárez <sup>3</sup> (in Hofmeister's laboratory).
C	27.80	27.29	25.13	36.08	43.20
H	3.48	3.64	3.88	5.32	5.47
N	2.32	2.58	2.11	2.89	5.37
S.....	5.30	4.85	4.26	1.85	4.29
Ba ..	22.70	21.90	18.35	14.38	

The substance gave a positive test with orcin in intensity resembling that of chondroitin sulfuric acid. On distillation with hydrochloric acid the presence of furfural was demonstrated by the aniline acetate test.

Efforts are being made to obtain the substance in a purer state. The results will be communicated in a subsequent report; the

<sup>1</sup> Alzona, F., *Biochem. Z.*, 1914, lxvi, 408.

<sup>2</sup> Levene, P. A., and Jacobs, W. A., *J. Exp. Med.*, 1908, x, 557. *Biochem. Z.*, 1909, xvi, 248.

<sup>3</sup> Levene and La Forge, *J. Biol. Chem.*, 1914, xviii, 239.

<sup>4</sup> López-Suárez, *Biochem. Z.*, 1913, lvi, 170.

present one deals with the nature of the nitrogenous component of the new conjugated acid.

The nitrogen-containing component was recognized as *d*-glucosamine. It was identified by the solubility of its hydrochloride, by the character and magnitude of its optical rotation, by the properties of its osazone, and finally by the conversion into chitonic acid.

This finding establishes the first chemical point of distinction between true mucin and mucoid. It is in harmony with the experience of F. Müller,<sup>10</sup> who obtained glucosamine on hydrolysis of salivary mucin.

Further work on mucoitin sulfuric acid (as we propose to name this substance) is now in progress. A survey of other mucins and mucoids is also contemplated.

#### EXPERIMENTAL PART.

*Preparation of the Crude Conjugated Sulfuric Acid.*—The mucous content of fresh pig's stomachs was dissolved in a 3 per cent solution of sodium hydroxide and allowed to stand for 48 hours. The solution was then rendered slightly acid by means of acetic acid, an excess of barium carbonate was added, and the mixture placed on the water bath, where it remained until the liquid acquired a very clear appearance. The precipitate was then removed by centrifugalization. Further treatment differed in individual experiments. In some the conjugated sulfuric acid was precipitated by means of glacial acetic acid, in others by means of lead acetate. The lead salts were converted into the free substance in the following manner. The salt was suspended in about ten parts of water, excess of barium carbonate added, and hydrogen sulfide passed through the solution. During this process the mixture was agitated by means of a mechanical stirrer, and the solution was kept at about 95°C. The filtrate from lead sulfide was concentrated under diminished pressure to about one-fourth of the original volume. To this solution an equal volume of 95 per cent alcohol was added, and in this manner the crude barium salt was obtained. The substance precipitated by means of glacial acetic acid was dissolved in water and precipi-

<sup>10</sup> Muller, F., *Z. Biol.*, 1901, xlii, 468.

tated by an equal volume of alcohol. The precipitate thus formed differed very little from the crude material obtained by the lead process.

*Purification of the Crude Material.*—The crude material contained impurities consisting principally of nucleic acid. In order to remove this the crude substance was dissolved in water, an excess of barium hydroxide solution was added, and the excess of barium hydroxide removed by a stream of carbonic acid gas. The barium salt of nucleic acid and barium carbonate were removed by centrifugalization. To the clear filtrate an equal volume of alcohol was added. The precipitate was redissolved, the solution again reprecipitated, and this was continued until the final precipitate gave an absolutely clear solution. About 80 per cent of the crude material is lost in the process of purification. For analysis the substances were dried under diminished pressure at the temperature of water vapor.

Sample I.

0.1368 gm. substance (Kjeldahl) neutralized 3.45 cc. 0.1 N HCl.

0.1524 " " gave 0.0200 gm. BaSO<sub>4</sub> (S determination).

0.1539 " " " 0.0562 " " (Ba " ).

Sample II.

0.0996 gm. substance gave 0.1318 gm. CO<sub>2</sub> and 0.0474 gm. H<sub>2</sub>O.

0.0952 " " (Kjeldahl) neutralized 1.97 cc. 0.1 N HCl.

0.0865 " " gave 0.0118 gm. BaSO<sub>4</sub> (S determination).

0.0908 " " " 0.0222 " " (Ba " ).

	Calculated for C <sub>23</sub> H <sub>41</sub> N <sub>2</sub> S <sub>2</sub> O <sub>29</sub> Ba <sub>2</sub> :	Found:	
		I.	II.
C.....	27.80		36.08
H.....	3.48		5.32
N.....	2.32	3.53	2.89
S.....	5.30	1.80	1.85
Ba.....	22.70	21.49	14.38

1 *Hydrolysis of the Conjugated Sulfuric Acid.*—20 gm. of the partially purified substance, taken up with 100 cc. of 20 per cent hydrochloric acid and 4.0 gm. of stannous chloride, were heated with a return condenser over flame for 7½ hours. The solution as diluted with an equal volume of water, hydrogen sulfide passed through the solution, and the filtrate concentrated under diminished pressure (approximately 15 mm.) nearly to dryness. A crystalline sediment formed in the flask. This was transferred

to a beaker by means of methyl alcohol. The substance was recrystallized out of dilute methyl alcohol. Unlike chondrosamine hydrochloride, the substance was insoluble in methyl alcohol, and crystallized in plates resembling those of glucosamine hydrochloride. The substance did not melt. It reduced Fehling's solution, and formed a glucosazone. For analysis it was dried in a vacuum desiccator.

0.0200 gm. substance in the Van Slyke amino apparatus gave 2.38 cc. N at 25° and 757 mm.

0.1500 gm. substance by the Volhard method required 6.86 cc. 0.1 N AgNO<sub>3</sub>.

	Calculated for C <sub>6</sub> H <sub>13</sub> O <sub>5</sub> NHCl:	Found:
N.....	6.51	6.57
Cl.....	16.45	16.23

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{\text{Initial: } +4.30^\circ \times 2.1862}{0.5 \times 0.2008 \times 1.039} = +90.1^\circ \quad [\alpha]_D^{20} = \frac{\text{Equilibrium: } +3.37^\circ \times 2.1862}{0.5 \times 0.2008 \times 1.039} = +70.63^\circ$$

*Oxidation of the Hexosamine.*—6 gm. of the sugar were dissolved in 25 cc. of water, 6.0 gm. of silver nitrite added, allowed to stand 6 hours, then another portion of 3.0 gm. of silver nitrite and the equivalent quantity of a 10 per cent hydrochloric acid solution were added. The mixture was allowed to stand over night. The solution then contained 0.0027 gm. of amino nitrogen. The excess of silver was removed by means of hydrogen sulfide. To the solution 15.0 gm. of bromine were added and allowed to stand 48 hours. The calcium salt of chitonic acid was then prepared in the usual way.

For analysis the calcium salt was dried in a vacuum desiccator at the temperature of water vapor.

0.0994 gm. substance gave 0.1212 gm. CO<sub>2</sub>, 0.0472 gm. H<sub>2</sub>O, and 0.0134 gm. CaO.

	Calculated for (C <sub>6</sub> H <sub>9</sub> O <sub>6</sub> )Ca+2H <sub>2</sub> O:	Found:
C.....	33.47	33.25
H.....	5.11	5.27
CaO.....	13.07	13.59

The optical rotation of the substance:

$$[\alpha]_D^{20} = \frac{+1.63^\circ \times 2.149}{1 \times 1.000 \times 1.010} = +34.67^\circ$$

Fischer<sup>11</sup> gives for the same substance  $[\alpha]_D^{19} = +32.8^\circ$ .

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<sup>11</sup> Fischer, E., and Tiemann, F., *Ber. chem. Ges.*, 1894, xxvii, 138.

## CEPHALIN. IV.\*

### PHENYL- AND NAPHTHYLUREIDOCEPHALIN.

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(Received for publication, June 1, 1916.)

In a previous communication we pointed out the discrepancy existing between the empirical analytical data of cephalin and the figures required by the theory, based on the assumption that the molecule contains only those components which have been identified on hydrolysis of the substance.

	C	H	N	P
Required.....	66.17	10.57	1.88	4.17
Found.....	60.00	9.38	1.68	4.27

At the time of that publication we were hopeful of obtaining convincing evidence as to the composition of cephalin by the conversion of the phosphatide into its tetrahydro derivative. However, little progress was made by that method. Various catalysts were prepared and found efficient for the reduction of unsaturated aliphatic compounds. Their efficiency was invariably disappointing when applied to the reduction of cephalin. Either the phosphatide itself or some impurity adhering to it acted as a catalytic poison, and after a brief interval of absorption of hydrogen the reaction stopped. Hence we were forced for the time to abandon the hope of preparing a satisfactory supply of the tetrahydro derivative.

At the same time it was attempted to approach the solution of the original problem from another angle. It has been conclusively demonstrated that cephalin contains a free amino group. *A priori* there is nothing to prevent this group from entering into the reactions characteristic of it. Hence cephalin, in a way,

\* Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913-14, xvi, 419; 1916, xxiv, 41, 111.



# THE UTILIZATION OF SUCROSE AND THE INVERTING POWER OF THE BLOOD SERUM AFTER PARENTERAL ADMINISTRATION OF SUCROSE.

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(Received for publication, May 20, 1916.)

It has been assumed that sucrose, which can be easily inverted in the alimentary tract, is eliminated quantitatively in the urine, when it is introduced into the animal body parenterally. According to the recent reports of Mendel and Mitchell (1905), Heilner, Mendel and Kleiner, and Hogan, the amount of sucrose recovered in the urine is less than that injected. Mendel and Kleiner concluded that, when sucrose was introduced parenterally into dogs and cats in doses of 1 or 2 gm. per kilo of body weight, the quantity excreted usually amounted to more than 65 per cent of that introduced. The results of other investigations are not far from this. Mendel and Kleiner, and Hogan were unable to cause any better utilization of sucrose by repeated injections. The fate of the part which cannot be recovered has not been determined. Jappelli suggested that part of the sucrose might be eliminated into the alimentary tract, and then be utilized, as is customary after oral administration. Reducing sugar is sometimes found in the urine, if the amount of sucrose injected is not too small, indicating that some of the sucrose injected is inverted somewhere in the animal body.

It is generally believed that in the animal body invertin can be found only in the mucous membrane of the intestine. Weinland claimed that repeated subcutaneous injections of sucrose cause an invertin to appear in the serum of puppies. He found, moreover, that the power to utilize sucrose increased with repeated injections. In his experiments, quantitative measurements were not attempted. Weinland explained his results by assuming that the invertin, which was found in the serum and caused the better utilization of injected sucrose, came from the intestine. Abderhalden and Brahm also believed that they could induce a production of invertin in the serum of dogs. This was considered to be an example of protective enzymes, which appear, according to Abderhalden, in serum, after introduction of substances normally foreign to the circulation. Their results were not constant. Subsequently, Abderhalden and Kapfberger stated that when the amount of sucrose injected was smaller, the results became constant. A single injection of 5 to 10 cc. of 10 per cent sucrose solution subcutaneously, or 2 cc. of 5 to 10 per cent solution intravenously was followed by the appearance of invertin in the serum in a short time—sometimes as soon as 15 minutes. The inverting power of the serum



remained for 14 days after the first injection. When this sucrase disappeared, a second injection caused the reappearance of the enzyme for another 14 days. Serum which had this inverting power could also change lactose, but not raffinose (experiments with dogs).

Upon the basis of the investigation of sucrose elimination and respiratory exchange after subcutaneous injection of this sugar, Heilner stated that part of the sucrose injected was oxidized in the animal body, assuming that it was first hydrolyzed by the invertin which was stated by others to appear. Pincussohn and Krause also tried to call forth invertin in the blood by injection of sucrose. In seventeen experiments with dogs and rabbits a positive result was found only once.

Kumagai did not succeed by Abderhalden's method; but after repeated injections of a much larger amount of sucrose, he found invertin constantly in the serum. His results seemed far more conclusive than those of Abderhalden and his collaborators. He stated that sucrose had the properties of an antigen and animals could be actively or passively immunized against it. Furthermore, serum which was active to sucrose could act upon the cleavage products and other kinds of sugar, such as milk sugar and galactose. Dextrose was reported transformed to levulose and from the one or the other of these simple sugars a disaccharide was synthesized. Röhmann and Kumagai believed that the product was lactose. Abderhalden and Wildermuth tried to confirm Kumagai's findings with rabbits. Serum which was active to sucrose was never active to any other kind of sugar. In Abderhalden's earlier experiments with dogs, such serum was also active towards lactose, but later it was reported to be negative in the case of rabbits. Inactivation and activation procedures were also negative. Abderhalden and Wildermuth confirmed the observation that a single injection of a very small amount of sucrose was enough to call forth invertin in serum. Abderhalden and Grigorescu, also working with dogs, obtained less constant results. These investigators ascribed such inconsistency to the variability in the occurrence of sucrase in the intestinal cells. The extreme rapidity of elimination of sucrose in some animals was also believed to account for the failure to provoke appearance of inverting enzyme in the blood. Abderhalden imagined that invertin in the serum has its origin in the intestine, because an animal in which the serum was first negative gave positive results in its action on sucrose after feeding this sugar.

Hogan also injected sucrose and lactose into dogs, but did not succeed in finding the corresponding inverting enzyme in the serum. On the other hand, Lombroso accepts the appearance of invertin in serum. Recently Röhmann, in whose laboratory Kumagai had worked, repeated Kumagai's experiments, but the results were far less constant. He says that the uncertainty which exists in the results of Abderhalden and his collaborators is also seen in his and Kumagai's results.

The question naturally arises whether, if an inverting enzyme is actually introduced into the blood, the utilization of parenterally injected sugar will be improved. La Franca reported that

when invertin is injected into the animal body before sucrose injection, the utilization of the latter is improved. This is interesting, when we compare the reports which insist on the appearance of sucrase with those which state that it is impossible to cause any better utilization of sucrose by repeated injection of this sugar.

It is conceivable that blood constituents may interfere with the action of inverting enzymes. Both Hildebrandt and Kumagai state that serum has no power to alter the activity of invertin. This is not in accord with the statements of Eriksson, and Griffin and Nelson.

In view of the numerous conflicting views reported above, I have, at the suggestion of Professor Lafayette B. Mendel, undertaken further investigations of some of the controverted problems.

The plan of the experiments included a reinvestigation of the extent of utilization of sucrose introduced parenterally, under a variety of conditions. In some cases large or small amounts of sugar were frequently injected to learn whether adaptation occurred or invertin was developed in the blood. In other cases active invertin was actually introduced into the blood stream to ascertain whether the enzyme would circulate so as to promote the utilization of sucrose in any way. The enzymatic activity of the blood serum of the experimental animals was repeatedly investigated.

#### *The Utilization of Sucrose after Parenteral Injection.*

Sucrose was repeatedly administered parenterally in adult dogs. They were kept in metabolism cages, the urine being collected in bottles containing toluene. The diet consisted of meat, bread, and salt. To prevent contamination of the urine with feces, bone meal (about 10 gm. a day) was mixed with the food. Water was given freely. The body weight of the dogs was nearly constant or decreased only a little during the experiments. The injections were made subcutaneously, intraperitoneally, or intravenously. For intravenous injection local anesthesia with cocaine was usually used, general anesthesia with ether being required only once (Dog B, Feb. 2).

The strength of the injected sugar solutions, estimated by the polariscope, was 10 per cent. Usually 50 cc. were given at one time. During March 15 to 29, 40 to 50 cc. of 40 per cent solution were employed for the injections in Dogs B and D.

The determination of sucrose in the urine was made by the polariscope with readings in Ventzke degrees, mercuric acetate being used to remove the coloring matters and levorotatory substances in the normal urine. 15 cc. of the saturated solution of mercuric acetate for 30 cc. of the urine were enough to gain practically the zero reading at the usual urine concentration. A known amount of sucrose added to the normal urine could be quantitatively determined by this method. At first both mercuric acetate and phosphotungstic acid were used successively, as Neuberg and Ishida suggested for removing protein cleavage products. Afterwards, mercuric acetate alone was found to be satisfactory, if the amount added was not too small and the mixture was not filtered too soon. Benedict's test was used for reducing sugar, Heller's test for protein.

Five invertin preparations were employed. Invertin Solutions I to IV were prepared from compressed yeast, but Invertin V was made from brewer's yeast. Following Hudson (1914), the yeast was washed and mixed with water and toluene in the proportion of 1 gm. yeast: 1 cc. water: 0.06 cc. toluene. It was allowed to autolyze 10 to 14 days. Protein was removed by neutral lead acetate and the excess of lead was removed by hydrogen sulfide. The filtrate was dialyzed for 2 days. The invertin solution was preserved with chloroform. The solution was clear, slightly yellow, and neutral in reaction. Only a very slight turbidity, if any, occurred on heating or adding nitric acid. The solution had no reducing power, but a very strong inverting one. The activity was tested before each trial. In addition, quantitative tests were made with each preparation. To 10 cc. of sucrose solution 1 to 2 cc. of invertin solution were added. In a few experiments the effect of addition of acid or alkali to the mixture was tested. The samples were kept at 37-40°C. for 5 to 20 minutes. Dilute ammonia was then added to stop the action of the invertin and complete the multirotation of the invert sugar (Hudson, 1909). The optical rotation (Ventzke scale) and the reducing power (Benedict's test) were then ascertained. The results of the tests of the invertin preparations are summarized in Table I.

Table II exhibits the numerous data on the utilization of sucrose in dogs after repeated injections of the sugar either alone or with simultaneous or previous injections of active invertin.

TABLE I.  
*Activity of the Invertin Preparations.*

Invertin.	Date.	Sucrose		Invertin	Reaction	Time in thermostat	Rotation	Reduction.
		cc.	per cent	cc		min.	V°.	
I.	Jan. 26	25	10	1	Neutral.	5	+29 90	+++
		"	"	1 (Boiled.)	"	5	+36 83	—
II.	Jan. 29	25	10	2	Neutral.	5	+30 85	+++
		"	"	2	"	10	+26 00	+++
		"	"	2 (Boiled.)	"	10	+35 25	—
	Feb. 16	25	10	2	"	20	+26 95	+++
		"	"	2 (Boiled.)	"	20	+35.15	—
		"	"	0 (2 cc. water.)	"	20	+35 10	—
III.	Feb. 7	25	10	2	Neutral.	5	+29 95	+++
		"	"	2 (Boiled.)	"	5	+35 70	—
	" 7	25	0 5	1	"	5	+1 65	+++
		"	"	1	Acid.*	5	+1 38	+++
		"	"	1	Alkaline.†	5	+1 86	—
		"	"	1 (Boiled.)	Neutral.	5	+1 90	—
	" 7	25	0 5	1	"	20	+1 13	+++
		"	"	1	Acid.*	20	+0 59	+++
		"	"	1	Alkaline.†	20	+1 89	—
		"	"	1 (Boiled.)	Neutral.	20	+1 88	—
IV.	Feb. 21	25	0 5	1	Neutral.	10	+1 70	+++
		"	"	1 (Boiled.)	"	10	+2 10	—
V.	Apr. 14	25	0 5	1	Neutral.	5	+1 05	+++
		"	"	1	Acid.*	5	+0 63	+++
		"	"	1 (Boiled.)	Neutral.	5	+1 85	—
	" 14	25	0 5	1	"	20	+0 08	+++
		"	"	1	Acid.*	20	-0 40	+++
		"	"	1 (Boiled.)	Neutral.	20	+1 83	—

\* Dilute acetic acid was added.

† Dilute ammonia was added.

TABLE II.

## Utilization of Sucrose Injected Parenterally.

Date.	Sucrose injection.			Invertin injection.			Urine.						Total sucrose recovered.	
	Time.	Amount.	Place.	Time.	Volume.	Place.	Date.	Time.	Vol- ume.	Sp gr	Reaction (litmus).	Sucrose.		
		gm.			cc.				cc.	10—		per cent	gm.	
Jan. 19	11.20 a.m.	4.97	Peritoneal cavity.				Jan. 19	12 a.m.	55.0		Acid.	1.020.56	4.0280.9	
							"	"	136.0	51	Alkaline.	2.523.43		
							"	1 p.m.	61.0	36	Acid.	0.050.03		
							"	9 a.m.	131.0	36	Alkaline.	0 0		
"	21 10.20 a.m.	1.97	" "				"	10 "	61.0	46	Acid.	0 0	4.0181.4	
							"	25 "	71.0	62	"	5.704.01		
							"	26 "	47.0	48	"	0 0		
							"	27 "	61.0	54	"	0.183.96		
26	12.20 a.m.	5.00	"	10.40 a.m. 12.20 p.m.	5.0 (I) 5.0 (I)	Subcutaneous. "	"	27 "	45.0	44	Neutral.	0 0	3.9670.1	
							"	27 "	221.0	42	Acid.	0.150.32		
							"	25 "	157.0	36	Alkaline.	2.133.31		
							"	26 "	237.5	36	Acid.	0 0		
"	26 12.30 p.m.	5.00	"				"	27 "	160.0	18	"	2.323.71	3.7174.1	
							"	28 "	310.0	38	"	0 0		
							"	29 "	218.0	12	"	1.031.05		
"	28 2.00 p.m.	1.97	"	12.30 p.m. 2.00 "	5.0 (II) 5.0 (II)	Subcutaneous. "	"	29 "	80.5	10	"	0 0		

Jan 31 10 00 a m	4 97	Peritoneal cavity.	9 00 a.m. 10 00 " 11 00 "	10 0 (II) 10 0 (II) 10 0 (II)	Subcutaneous " "	Feb. 1 " 1	9 a.m. 1 p.m.	191 0 201 0	37 18	Acid "	1 72 3 33 0 0	3 33 67.0
Feb 2 11 10 a m	4 98	"	11 00 "	19 0 (II)	V. jugularis ext.	" 3	9 a.m.	302 0	27	"	1 03 3 10	3 10 64 0
" 4 5 40 p m	4 98	"	12 00 "	25 0 (II)	Subcutaneous	" 3 " 1	8 p.m. 9 a.m.	117 0 220 0	22 26	" Neutral.	0 06 0 09 0 0	3 10 64 0
" 4 5 40 p m	4 98	"	5 30 p.m.	40 5 (III)	V jugularis ext	" 5 " 6	9 a.m. 5 p.m. 10 a.m.	196 0 163 0 180 0	25 20 26	Neutral Acid "	1 57 3 07 0 0 0 0	3 07 63 0
" 7 11 50 a m	4 97	"	"	"	"	" 8 " 8 " 8	9 " 3 p.m. 5 "	300 0 19 0 45 0	23 20 17	" " "	1 00 3 29 0 0 0 0	3 29 66 1
" 8 6 00 p.m.	4 97	"	"	"	"	" 9 " 9 " 10	9 a.m. 11 " 9 "	171 0 63 0 110 0	29 26 27	" " "	1 85 3 16 0 45 0 28 0 0	3 41 69 2
" 11 3 00 p m	4 97	"	"	"	"	" 12 " 13	9 " 10 "	255 0 203 0	30 36	" "	1 30 3 32 0 0	3 32 66 8
" 14 12 50 p m	5 00	"	"	"	"	" 14 " 15	7 p.m. 9 a.m.	129 0 56 0	32 49	" "	3 03 3 91 0 0	3 91 78 2
" 16 4 05 p m	5 00	V jugularis ext	4 00 p.m.	45.0 (II) (Boiled.)	V. jugularis ext.	" 17 " 18*	9 " 9 "	150 0 215 0	38 36	" Neutral	2 59 3 98 0 0	3 98 79 6

\* Protein %.

TABLE II—Continued.

Date.	Sucrose injection			Invertin injection			Urine							
	Time	Amount	Place	Time	Volume	Place	Date.	Time	Vol- ume cc	Sp gr	Reaction (litmus)	Sucrose		Total sucrose recovered.
		gm.			cc							per cent	gm.	per cent
Feb. 18	3 30 p.m.	5.00	V. jugularis ext.	3 20 p.m.	45.0 (IV)	V. jugularis ext.	Feb. 19*	9 a.m.	219.0	27	Alkaline	1.51	3.30	3.30
"	3 00 p.m.	5.00	" "	2 50 "	45.0 (IV)	" "	" 19	" 11	93.5	40	Acid.	0	0	
"	3 00 p.m.	5.00	" "	2 50 "	45.0 (IV)	" "	" 20	" 11	116.0	40	"	1.94	3.21	3.21
"	12.00 a.m.	5.00	V. saphena mag.	11.50 a.m.	100.0 (IV)	V. saphena mag.	" 20	" 12	145.0	30	"	0	0	
Mar. 1	1.00 p.m.	5.00	Peritoneal cavity.				" 23	9 "	149.0	38	Alkaline.	1.40	2.09	2.09
"	15-29	20.0 8 times.	" "				" 24	9 "	499.0	26	Acid.	0	0	
"	30 5.00 p.m.	5.00	" "				Mar. 2†	9 "	207.0	41	"	1.48	3.06	3.06
"	30 5.00 p.m.	5.00	" "				" 3†	10 "	135.0	45	"	0	0	
Apr. 3	11 30 a.m.	5.00	" "				" 15-30§							4.19
"	30 5.00 p.m.	5.00	" "				" 31	10 a.m.	314.5	42	"	1.31	1.19	
"	30 5.00 p.m.	5.00	" "				Apr. 1	3 p.m.	200.0	24	"	0	0	4.19
"	30 5.00 p.m.	5.00	" "				" 4	12 a.m.	263.0	11	Neutral.	1.57	1.12	4.12
"	30 5.00 p.m.	5.00	" "				" 5	12 "	378.0	27	Acid.	0	0	

Jan. 31	9.30 a.m.	4.97	Peritoneal cavity.			Feb. 1 " 2†	9 a.m. 9 "	112.0 67.0	48 42	Acid. "	3.17 0	3.55 0	3.55 71.4
Feb. 2	12.30 p.m.	4.98	"	11.30 a.m. 12.20 p.m.	20.0 (II) 20.0 (II)	Subcutaneous. "	3 4	5 p.m. 9 a.m.	50 45	" "	3.57 0	3.50 0	3.50 70.2
"	4 7.45 p.m.	4.98	"	6.30 "	55.0 (III)	"	5 6	12 10	33 36	Neutral. Alkaline.	2.44 0	3.95 0	3.95 79.3
"	7 11.35 a.m.	4.97	V. jugularis ext.	11.30 a.m.	45.0 (III)	V. jugularis ext.	8 8 9	9 7 p.m. 9 a.m.	28 37 36	Acid. " Alkaline.	0.79 0 0	1.52 0 0	1.52 30.5
"	9 12.05 p.m.	4.97	"	12.00 "	45.0 (III)	"	9 10	5 p.m. 9 a.m.	30 42	Acid. Alkaline.	1.37 0	1.68 0	1.68 33.7
"	11 2.00 p.m.	4.97	Peritoneal cavity.				12 13	9 10	44 37	Neutral. Acid.	3.60 0	3.60 0	3.60 72.3
"	14 1.50 p.m.	1.00	"										
"	3.20 p.m.	1.00	"										
"	4.50 p.m.	1.00	"										
"	5.50 p.m.	1.00	"										
"	6.50 p.m.	1.00	"										
"							15† 15	9 12	66 46	" "	5.67 0	3.74 0	3.74 74.4

\* Protein =.

† Reduction +.

‡ Reduction =.

§ Sometimes reduction +.



TABLE II—Concluded.

TABLE II—Continued.

Date.	Sucrose injection.			Invertin injection			Urine.					Total sucrose recovered	
	Time.	Amount.	Place.	Time	Volume	Place	Date	Time	Vol- ume	Sp gr	Reaction (litmus).	per cent	gm.
Jan. 31	9.40 a.m.	4.97	Peritoneal cavity.		cc.		Feb. 1*	9 a.m.	215.0	48	Acid.	1.803	88
							" 3*	9 "	246.0	42	"	0	0
Feb. 9	12.35 p.m.	4.97	V. jugularis ext.	12.30 p.m.	45.0 (III)	V. jugularis ext.	" 9	7 p.m.	140.0	18	Alkaline.	0.821	15
" 11	2.30 p.m.	4.97	" "				" 10	2 "	168.0	32	Acid.	0	0
" 25	12.10 p.m.	5.00	Peritoneal cavity.	11.50 a.m.	45.0 (III) (Boiled.)	Peritoneal cavity.	" 12	9 a.m.	175.0	29	Alkaline.	2.354	11
							" 13	10 "	180.0	40	Acid.	0	0
Mar. 2	7.00 p.m.	4.91	Subcutane- ous.				" 26	9 "	273.0	34	Neutral.	1.604	39
" 15-29		16.04 18.04	Peritoneal cavity.				" 26	5 p.m.	131.0	17	Acid.	0	0
" 30	4.50 p.m.	5.00	" "				Mar. 3*	9 a.m.	79.5	48	"	3.903	10
							" 4	9 "	65.0	50	Neutral.	0.700	46
Apr. 3	12.00 a.m.	5.00	" "				" 4	5 p.m.	120.0	31	Acid.	0	0
" 6	11.00 a.m.	5.00	" "	10.55 a.m.	90.0 (V)	V. brachialis.	" 15-30†						
							" 31	4 p.m.	273.5	30	"	1.624	42
Apr. 3	12.00 a.m.	5.00	" "				Apr. 2	12 a.m.	123.0	32	"	0	0
" 6	11.00 a.m.	5.00	" "				" 4	12 "	230.0	39	"	1.924	41
							" 5	12 "	145.0	32	"	0	0
" 7							" 7*	11 "	237.0	10	Alkaline.	1.333	15
" 8							" 8	12 "	126.0	10		0	0
													3.1563.0

Dog D, 7.5 Kg.

Dog E, ♂ 12.1 kg.	Mar. 30	4.40 p.m.	5.00	Peritoneal cavity.					Apr. 1 " 2 " 3	11 a.m. " " " "	215.0 100.0 137.0	35 33 45	Acid. " Neutral.	1.392.99 0.240.23 0 0	3.2264.4
	Apr. 3	12.00 a.m.	5.00	" "					" 5* " 6	9 12	323.0 192.0	48 51	Acid. Neutral.	1.173.79 0 0	3.7975.8

\* Reduction =.

† Sometimes reduction +.

‡ 4 times.

*Injection of Sucrose Alone.*

A résumé of the essential features with respect to the repetition of the sugar injections and their effect on the utilization is given in Tables III and IV.

TABLE III.  
*Data Regarding Injections of Sucrose and Invertin*

Dog	Duration of experiment	Injection of sucrose alone		Injection of sucrose and invertin	
		Sucrose per kg body weight	No of injections	Sucrose per kg body weight	No of injections
	<i>days</i>	<i>gm</i>		<i>gm.</i>	
A	6	0.4	2	0.4	1
B	70	0.4	9	0.4	8
		1.5	8		
C	15	0.9	3	0.9	4
D	67	0.7	5	0.7	3
		2.1	4		
		2.4	4		
E	5	0.4	2		

TABLE IV.

*Recovery of Sucrose (in Urine) after Repeated Injections (Sucrose Alone Injected).*

Dog	Maximum	Minimum	Average	After first injection	After last injection
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
A	81.4	80.9	81.2	80.9	81.4
B	83.8	61.2	72.9	73.7	82.4
C	74.4	71.4	72.7	71.4	74.4
D	88.4	72.5	81.8	78.0	88.2
E	75.8	64.4	70.1	64.4	75.8

Average of 21 injections = 75.5 per cent.

The tables show clearly that the parenteral utilization of sucrose was not improved by the repeated injections. This agrees with the reports of Mendel and Kleiner, and Hogan. Elimination of sucrose usually ends within 24 hours after the injection. The same degree of utilization of sucrose occurred after the different paths—intraperitoneal, intravenous, or subcutaneous—of administration. There was no evidence of interference with the normal renal function. Usually the volume of the urine was undiminished and protein did not appear. When the amount of sugar injected was 0.4 to 0.9 gm. per kilo of body weight, more than a trace of reducing substance was never found. With larger amounts of sugar (1.5 to 2.4 gm. per kilo of body weight) a detectable amount of reducing sugar was sometimes found in the urine. The general condition of the dogs was not affected after sugar injections, except for a slight transitory fever after two or three of the injections.

*Injection of Invertin and Sucrose.*

Invertin and sucrose were injected in succession, in sixteen experiments, as shown by the protocols recorded in Table II. A better utilization of sucrose, particularly after intravenous injections, was gained as follows:

Dog.	Invertin injected.	Sugar recovered.
	cc.	per cent
B.....	100 (IV)	41.8
C.....	45 (III)	30.5
C.....	45 (III)	33.7
D.....	45 (III)	23.1

A large amount of invertin was required to produce noteworthy effects. When 40.5 cc. (III) to 45.0 cc. (IV) of invertin were injected in Dog B, 63 to 66 per cent of sucrose was recovered. These values are scarcely lower than that sometimes obtained without the invertin injection. When invertin and sucrose were administered by different paths—i.e., one intravenously, the other intraperitoneally—the utilization was only a little better than in the control without invertin, or else no difference at all was seen.

The protocols in general indicate that the enzyme of yeast is able to invert sucrose in the circulation. To produce such a result the enzyme and its substrate must, however, be brought together in the organism. For this reason, the utilization is improved when the enzyme is introduced directly and almost simultaneously into the circulation with the sugar; but after subcutaneous administration of invertin, whereby it reaches the rapidly circulating sugar slowly, if at all, little advantage is noted. Even when an improved utilization was noted, reducing sugar was not found in the urine. La Franca reported much better utilization than was found in my experiments. In his paper there is no description of the activity of the invertin he used.

On the 3rd day after the intravenous injection of invertin the utilization of sucrose was the same as before the injection of the invertin. (Dog C, 33.7 per cent on Feb. 9, but 72.3 per cent on Feb. 11; Dog D, 23.1 per cent on Feb. 9, but 82.1 per cent on Feb. 11.) This agrees with the result that the serum taken 20 to 24 hours after the intravenous injection of invertin no longer shows an inverting power (compare Tables VIII and IX). It is evident, therefore, that the inverting power after injection of invertin is not long retained.

*Does the Blood Acquire Inverting Power after Parenteral Injection of Sucrose?*

Serum was examined by the polariscope, before and after incubating with sucrose. A 2.5 cm. tube was used when the amount of serum was small; and a 2 dm. tube, when more serum was available. Blood was taken by venopuncture. After 1 or 2 hours serum was separated by centrifugation. For the 2.5 cm. tube 1 cc. serum + 1 cc. 10 per cent sucrose + 2 cc. water + 1 drop of toluene were used. This mixture, in tightly stoppered tubes, was kept 40 to 48 hours at 37–40°C. The protein was removed by the colloidal iron method (1 cc. colloidal iron and 0.1 gm. powdered  $\text{Na}_2\text{SO}_4$ ) before the filtrate was examined with the polariscope. For this series the final volume represented a dilution of the serum to five times the original. For the 2 dm. tube, 3 cc. serum + 3 cc. 10 per cent sucrose + 6 cc. water

+ 3 drops of toluene were kept in the thermostat under the same conditions. Then 3 cc. colloidal iron, 0.3 gm.  $\text{Na}_2\text{SO}_4$ , and 15 cc. water were added. The final volume was ten times that of the original serum. An attempt was also made to "activate" the invertin by making the solution neutral or very slightly acid, by addition of acetic acid of about 0.025 N and 0.013 N strength. In the tables, the different degrees of acidity are designated as "acid (stronger)" and "acid (weaker)." The procedure, when acid was added, was as follows: 1 cc. serum + 1 cc. 10 per cent sucrose + 1 cc. acid (weaker) + 1 cc. water + 1 drop of toluene (for the 2.5 cm. tube), or 3 cc. serum + 3 cc. 10 per cent sucrose + 3 cc. acid (stronger) + 3 cc. water + 3 drops of toluene (for the 2 dm. tube). The reaction of the mixture was neutral, or very slightly acid to litmus. As a control, the same mixture was examined at once without incubation. When 0.9 per cent NaCl was used instead of serum, sometimes a very slight decrease of rotation and reducing substance were noted after incubating. When the same acid was added to serum, the change, which was due to the acid alone, never appeared. Rotations are expressed in degrees of the Ventzke scale. In numerous preliminary trials, it was shown that the removal of protein by colloidal iron did not interfere with the determination of sucrose and its digestion products. The experimental error in these trials was not greater than  $0.1^\circ\text{V}$ . Reducing power was tested by Benedict's method, and controlled by comparison with the normal serum which contains a small amount of dextrose. The test was made by adding three or six drops (according as the dilution of the serum was five or ten times) to 5 cc. of the reagent. These proportions gave no visible reduction with normal serum. The dogs used had served in the experiments recorded in Table II. The results are summarized in Table V.

Five rabbits were also used. Sucrose was always injected in 40 per cent solution.

Rabbit A, used as a control without injection.

Rabbit B, 2.3 kg. Sucrose was injected *subcutaneously*; viz., 4 gm. on Mar. 17, 6 gm. on Mar. 18, 20, and 22, 8 gm. on Mar. 25, 27, and 29, 16 gm. on Apr. 2.

Rabbit C, 2.6 kg. Sucrose was injected *intraperitoneally*; viz., 6 gm. on Mar. 18, 20, and 22, 8 gm. on Mar. 25, 27, 29, and Apr. 2.

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Rabbit D, 2.5 kg. *Sucrose* was injected *intraperitoneally*; viz., 4 gm. on Mar. 17, 6 gm. on Mar. 18, 20, and 22, 8 gm. on Mar. 25, 27, and 29.

Rabbit E, 2.8 kg. *Sucrose* was injected *intravenously*; viz., 4 gm. on Mar. 17, 6 gm. on Mar. 18, 20, and 22, 8 gm. on Mar. 25, 27, 29, and Apr. 2.

Bread, corn, carrots, and cabbage were fed. The sucrose injection caused no noticeable disturbance. The urine sometimes contained a small amount of reducing sugar, but usually none. The records are given in Table VI.

The records of Tables V and VI show that the serum had no power to decrease the rotation of serum-sucrose mixtures; i.e., to invert sucrose. The dogs here used were the same ones, in which invertin was injected. It is scarcely to be assumed that previous injection of yeast invertin prevented the appearance of a "protective" sucrase.

Only a few experiments were made to see if the serum, which is taken after sucrose injection, has also the power to change dextrose into levulose and synthesize lactose from these. The method was the same as above mentioned (with the 2.5 cm. tube). 10 per cent dextrose solution and Seliwanoff's test were used instead of sucrose solution and Benedict's test. The results were negative (see Table VII).

TABLE VII.  
*Action of Serum upon Dextrose.*

Animal.		Date.	Examined immediately.		Examined after incubating 40 to 48 hrs. at 37-40° C.	
			Rotation.	Seliwanoff's test.	Rotation.	Seliwanoff's test.
Dog.	B	Mar. 30	+0.63	—	+0.63	—
	D	" 30	+0.63	—	+0.63	—
Rabbit.	B	Mar. 31	+0.63	—	+0.63	—
		Apr. 2	+0.70	—	+0.60	—
	C	" 2	+0.70	—	+0.63	—
	D	Mar. 31	+0.63	—	+0.63	—
	E	Apr. 1	+0.65	—	+0.63	—

*Examination of the Blood Serum and Urine for Invertin after Injection of Yeast Invertin.*

The inverting power of the serum taken at various intervals after invertin injection was examined by the method already described (2.5 cm. tubes).

Urine was collected during 48 hours after the invertin injections, and examined for invertin, as follows: 20 cc. urine + 10 cc. 10 per cent sucrose + 1 cc. water + 3 drops of toluene were incubated 24 hours at 37-40°C. To 25 cc. of the mixture 10 cc. of the mercuric acetate solution were added. The filtrate was then examined polariscopically. As a control, boiled urine or water was used instead of urine; and by adding 1 cc. of invertin instead of 1 cc. of water it was demonstrated that if invertin was present in the urine, it could be found by this method. All portions of the urine were acid, except the second sample from Dog D. In some experiments two drops of dilute acetic acid were added to increase the activity of invertin, if any was present. The results are given in Tables VIII and IX.

TABLE VIII.

*Dog. B. Mar. 10, 100 Cc. Invertin (IV) Were Injected into V. Femoralis Serum Tests.*

Serum taken.		Examined immediately.		Examined after incubating 40 hrs. at 37-40° C			
		Serum + sucrose.		Serum + sucrose		0.9 per cent NaCl + sucrose	
		Alone	+ Acid (strong- er)	Alone	+ Acid (strong- er).	Alone	+ Acid (strong- er)
Just before injection.	Rotation V°	+0 80	+0 78	+0 85	+0 79		
	Reduction	—	—	—	—		
10 minutes after injection.	Rotation V°	+0 80	+0 79	+0 56	-0 28	+0 80	+0 75
	Reduction	—	±	+	+++	—	+
1 hour after injection.	Rotation V°	+0 85	+0 83	+0 73	-0 04		
	Reduction	—	—	±	+++		
20 hours after injection.	Rotation V°	+0 81	+0 80	+0 80	+0 81	+0 78	+0 70
	Reduction	—	—	—	—	—	+



# Urine Tests.

Urine of		Urine + sucrose					Water + sucrose + Invertin (iv)
		Alone	Alone (boiled urine).	+ Acid	+ Acid (boiled urine)	+ Invertin (iv)	
1st day.	Rotation V° Reduction	+8 55 —	+8 48 —	+8 50 —	+8 50 —	-1 60 +++	-1 80 +++
2nd day	Rotation V° Reduction	+8 70 —	+8 70 —	+8 77 —	+8 70 —	+6 80 +++	-2 65 +++

TABLE IX.

*Dog D Mar 6, 45 Cc Invertin (IV) Were Injected into V. Femoralis Serum Tests.*

Serum taken		Examined immediately		Examined after incubating 49 hrs at 37-40° C			
		Serum + sucrose alone	0.9 per cent NaCl + sucrose alone	Serum + sucrose		0.9 per cent NaCl + sucrose	
				Alone	+ Acid (stronger)	Alone	+ Acid (stronger)
10 minutes after injection	Rotation V° Reduction	+0 78 =	+0 80 —	+0 63 +	-0 16 +++	+0 75 —	+0 75 =
1 hour after injection	Rotation V° Reduction	+0 82 —	+0 80 —	+0 69 +	-0 16 +++		
24 hours after injection	Rotation V° Reduction	+0 78 —	+0 75 —	+0 80 —	+0 79 —		+0 75 =

# Urine Tests.

Urine of 48 hrs after injection		Urine + sucrose					Water + sucrose + Invertin (iv)
		Alone	Alone (boiled urine)	+ Acid	+ Acid (boiled urine)	+ Invertin (iv)	
1	Rotation V° Reduction	+8 80 —	+8 80 —	+8 80 —	+8 80 —	+1 90 +++	-2 60 +++
2*	Rotation V° Reduction			+8 73 —	+8 73 —	+5 15 +++	+1 80 +++
3	Rotation V° Reduction	+8 78 —	+8 75 —	+8 73 —	+8 75 —	-0 70 +++	-2 15 +++

\* As this portion was alkaline, two drops of acetic acid were always added

These experiments indicate that the serum taken 10 minutes or 1 hour after the end of the invertin injection had an inverting power. When dilute acid was added, this power increased markedly, but it was no longer present in the serum collected 20 to 24 hours after injection. Invertin was not eliminated in the urine.

*The Effect of Serum or Blood upon the Activity of Yeast or Intestinal Invertin (in Vitro).*

It is conceivable that blood may interfere in some way with the demonstration of the presence of sucrase. Accordingly this problem was investigated. 10 cc. of defibrinated pig's blood (or as a control 0.9 per cent NaCl) + 2 cc. yeast invertin (IV) (as a control, boiled invertin or 0.9 per cent NaCl) + 5 cc. 10 per cent sucrose were incubated 40 minutes at 37–40°C. 53 cc. of distilled water, 30 cc. of colloidal iron, and 0.5 gm. of powdered  $\text{Na}_2\text{SO}_4$  were added successively. To 30 cc. of the filtrate 1 cc. of colloidal iron was added again. After addition of dilute ammonia to 25 cc. of the filtrate the rotation and reducing power were tested (2 dm. tubes). Invertin and sucrose were added either together or successively after an interval (see Table X).

The results show that, though the invertin could act in defibrinated pig's blood, its efficiency was decreased markedly. When invertin and blood were kept together a little while before the sucrose addition, the decrease of the activity of the invertin seemed to be larger.

In another series (Table XI), the activity of yeast invertin (IV) added to normal dog serum was examined by the same method as that used in the investigation of the serum after sucrose or invertin injections (p. 534). The 2.5 cm. tubes were used. Two invertin solutions, diluted ten and twenty times, were prepared. In the table one is described as "invertin (stronger)," the other as "invertin (weaker)." The mixing was done as follows: 1 cc. serum + 1 cc. 10 per cent sucrose + 1 cc. acid (stronger) + 1 cc. invertin (weaker) + 1 drop of toluene. For analysis immediately or after incubation 1 cc. of colloidal iron and 0.1 gm. of powdered  $\text{Na}_2\text{SO}_4$  were added.

TABLE X.

*Activity of Invertin in Defibrinated Pig's Blood.*

	Blood + sucrose.			0.9 per cent NaCl + sucrose + invertin (2 cc.).	The addition of the invertin and sucrose was made at the same time.
	+ Invertin (2 cc.).	+ Boiled invertin (2 cc.).	Alone.		
Rotation V°	-0.05	+1.95	+1.95	-0.45	
Reduction	+++	—	—	+++	

	Blood + sucrose.			0.9 per cent NaCl + sucrose.		The invertin was added and the mixture was kept 15 minutes in the thermostat before the sucrose addition.
	Alone.	+ In- vertin (2 cc.).	+ In- vertin (1 cc.).	+ In- vertin (2 cc.).	+ In- vertin (1 cc.).	
Rotation V°	+1 93	+0.50	+1.30	-0.48	+0.05	
Reduction	—	+++	+++	+++	+++	

	Blood + sucrose.			0.9 per cent NaCl + sucrose + invertin* (2 cc.).
	+ Invertin* (2 cc.).	+ Invertin† (2 cc.).	+ Invertin‡ (2 cc.).	
Rotation V°	+0.50	+0.25	+0.55	-0.45
Reduction	+++	+++	+++	+++

\* The invertin was first added. After keeping 15 minutes in the thermostat, the sucrose was added.

† The invertin and sucrose were added at the same time.

‡ The invertin was first added. After keeping 1 hour in room temperature and 15 minutes in the thermostat, the sucrose was added.

To study further the effect of serum upon intestinal sucrase, serum and intestinal extracts, prepared from the same dog (D) by the method described by Mendel and Mitchell (1907), were tested together. The scrapings of the mucous membrane of the whole intestine were extracted for 24 hours with double their volume of water. From this very slightly acid extract, solutions, diluted three and six times respectively, were made—"invertin (stronger)" and "invertin (weaker)."

The results in Table XI show that yeast and intestinal extracts can invert sucrose in the presence of dog serum, although their activity is decreased very much, compared with the control trials without serum. When dilute acid was added the enzymatic reaction increased markedly. In control experiments with 0.9 per

TABLE XI.

## Activity of Invertin in Dog Serum.

		Examined after incubating 10 hrs at 37-40°C.									
		Examined immediately.		+ Acid (weaker).		+ Acid (stronger).		Without acid.		+ Acid (weaker)	+ Acid (stronger)
		Neither acid nor invertin added.	+ Acid (stronger)	+ Invertin (weaker).	+ Invertin (stronger).	- Invertin (weaker).	- Invertin (stronger).	+ Invertin (weaker).	+ Invertin (stronger).	Without invertin.	Neither acid nor invertin added.
Activity of yeast invertin.	Normal dog serum + sucrose.	+0 80	+0 75	-0 11	-0 11	-0 11	-0 15	+0 70	+0 63	+0 75	+0 75
	0 9 per cent NaCl + sucrose.	-	±	+++	+++	+++	+++	+	+	-	-
	Serum of Dog D + sucrose.	+0 78	+0 78	+0 05	-0 03	-0 05	-0 15	0 0	-0 05	+0 75	+0 75
	0 9 per cent NaCl + sucrose.	-	±	+++	+++	+++	+++	+++	+++	-	-
Activity of intestinal invertin of Dog D	Normal dog serum + sucrose.	+0 83	+0 82	+0 08	+0 18	+0 25	-0 75	+0 78	+0 75	+0 88	+0 88
	0 9 per cent NaCl + sucrose.	-	±	+++	+++	+++	+++	-	±	-	-
	Serum of Dog D + sucrose.			+0 05	-0 58	-0 43	-1 00	+0 13	+0 83		
	0 9 per cent NaCl + sucrose.			+++	+++	+++	+++	+++	+++		
Activity of intestinal invertin of Dog D	Normal dog serum + sucrose.	+0 80	+0 80	+0 45	+1 35	+0 75	+0 33	+0 45	+2 00		
	0 9 per cent NaCl + sucrose.	-	-	+++	+++	+++	+++	+++	+++		
	Serum of Dog D + sucrose.			+0 60	+0 75	+0 63	+0 28	-0 65	-1 00		
	0 9 per cent NaCl + sucrose.			±	+++	±	+	+++	+++		

cent NaCl, the potency of yeast invertin increased with acid addition, but the activity of intestinal invertin decreased with the same acid addition. Two explanations suggest themselves. (1) The yeast invertin solution was neutral, but the intestinal invertin solution was very slightly acid. Therefore acid addition to the mixture of the physiological solution and intestinal invertin solution caused an augmented acidity, which may have inhibited the activity of the invertin. (2) The optimum point of the reaction of the medium may be different for these two kinds of invertin. It is clear, though, that invertin from both sources can invert sucrose in serum.

#### SUMMARY AND DISCUSSION.

In correspondence with earlier observations in this laboratory, sucrose, administered parenterally to dogs, was not eliminated quantitatively in the urine. The amount recovered varied considerably, averaging 76 per cent when sucrose was injected in doses of 0.4 to 0.9 gm. per kilo of body weight. The elimination was usually concluded within 24 hours. The degree of utilization was essentially the same for the different paths of parenteral introduction of the sugar.

The apparent utilization of a small part of the sucrose, as judged by its failure to be excreted by the kidneys, may be due to the presence or rapid production of sucrase in the blood. Other explanations, such as the assumption of an excretion of part of the sugar into the intestine and the reabsorption of the products of its inversion there, are discussed above.

The utilization of the sucrose was not essentially changed after many repeated injections of sugar. This likewise is in accord with earlier experience in this laboratory and is not what one would expect if parenteral introduction of a disaccharide leads to any abundant formation of protective enzymes which can hydrolyze it. It is possible, of course, that the actual amount of inverting enzyme brought into the circulation by the parenteral injections is too small to affect the utilization of large amounts of a sugar that can be rapidly eliminated. It is also possible that invertin is produced in considerable amount, but cannot act effectively under the conditions prevailing in the circulation. These questions will be considered below.

Experiments *in vitro* showed that the inverting power of active yeast or intestinal extracts was not lost in the presence of defibrinated blood or serum, although it was considerably decreased. Addition of acid facilitated the invertin reaction and therefore was carried out in some of the experiments with serum.

That the activity of sucrase is retained in the circulation itself was shown by the results of injecting solutions of active yeast invertin; for under such conditions injected sucrose failed to reappear in as large amounts as usual. The better utilization was not due to any failure of the kidney functions in excreting sucrose. The invertin can be demonstrated in the blood after injection of such an enzyme extract. It soon disappears from the circulation, and can no longer be detected in the serum at the end of 20 to 24 hours. The better utilization of sucrose thereupon does not continue to be manifested. Invertin was not excreted into the urine in my experiments.

The inverting power of the blood serum, after intravenous injections of yeast invertin, under the experimental conditions indicated in Tables VIII and IX, is shown by the following decreases in the optical rotation of the sucrose-serum mixtures:  $V^{\circ}$  0.24, 0.12, 0.15, 0.13 in 40 hours. It is almost impossible to compare the data given by other investigators, owing to the great diversity of conditions and methods employed. From published protocols the following are selected for consideration.

Abderhalden and Kapfberger: 0.5 cc. serum + 0.5 cc. 10 per cent sucrose + 7 cc. 0.9 per cent NaCl. Rotation was examined without removing protein (length of the tube not given). Decrease of rotation was about  $0.06^{\circ}$ ,  $0.15^{\circ}$ ,  $0.42^{\circ}$ , etc., in 15 to 99 hours.

Abderhalden and Wildermuth: 1 cc. serum + 1 cc. 5 per cent sucrose. Protein was not removed (length of the tube not given). Decrease of rotation was about  $0.08^{\circ}$ ,  $0.1^{\circ}$ ,  $0.17^{\circ}$ ,  $0.18^{\circ}$ , etc., in 32 to 49 hours.

Kumagai: 2.5 cc. serum + 2.5 cc. 5 per cent sucrose + 10 cc. water. After protein was removed (1 gm. sodium acetate and 1 cc. 30 per cent ferric chloride), rotation was tested with a 1 dm. tube. Decrease of rotation was about 10', 15', 42',  $1^{\circ} 30'$ ,  $1^{\circ} 50'$ , etc., in 20 to 48 hours.

Röhmman: 0.5 cc. serum + 0.5 cc. 10 per cent sucrose + 9 cc. water. After protein was removed (0.2 to 0.5 gm. sodium acetate and 4 to 5 drops of 30 per cent ferric chloride), rotation was tested with a 1 dm. tube. Decrease of rotation was about 12', 18', 20', 44', 51', etc., in 24 to 48 hours.



# A COMPARISON OF THE RESULTS OBTAINED BY THE COLORIMETRIC AND GRAVIMETRIC DETERMI- NATIONS OF CHOLESTEROL.

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It has been observed in the course of a considerable number of cholesterol determinations made by the colorimetric method of Autenrieth and Funk,<sup>1</sup> and the digitonin method of Windaus<sup>2</sup> (gravimetric) that the averages of values obtained were considerably higher in the colorimetric than in the gravimetric analyses. When this fact became appreciated, check determinations were undertaken on the same specimens of blood, and it soon was evident that there was a real source of error in one of the methods, since there was invariably a difference of as much as 10 per cent and often more, the colorimetric results being higher. In a new modification of the colorimetric determination recently published, Bloor<sup>3</sup> has claimed still higher results than those obtained by the Autenrieth-Funk method, by an average of 20 per cent. Grave doubts were thus cast on the reliability of the digitonin method. Since, however, this is the only available means of determining separately the free and combined forms of cholesterol it seemed highly desirable to undertake a critical study of the two procedures and to attempt to locate, and, if possible, to rectify the error. To this end it was necessary to study both the actual technique of the determination and the method of extraction of the cholesterol from the tissues.

In the literature there is comparatively little which might throw any light upon the question. Autenrieth and Funk<sup>1</sup> quote one analysis in which

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<sup>1</sup> Autenrieth, W , and Funk, A , *Munch med Woch* , 1913, lv, 1243

<sup>2</sup> Windaus, A , *Z physiol Chem* , 1910, lxx, 110

<sup>3</sup> Bloor, W R , *J Biol Chem* , 1916, cxiv, 227.



extraction flask, by means of which he has obtained good results. A simple and at the same time a most effective way to obtain a perfectly colorless solution is to unite the chloroform extracts and shake them out once or twice with distilled water in a separatory funnel, and then dehydrate with sodium sulfate. The water quickly and completely removes all color from the chloroform.

While the Autenrieth-Funk method has been used throughout as the simplest and most accurate of the colorimetric methods, the method recently described by Bloor<sup>3</sup> has been run parallel with it in several analyses. Bloor's statement that his method gives considerably higher results than the Autenrieth-Funk method has been entirely confirmed, but in the light of what is to follow, it appears that to whatever cause this increase is due, it is not due to cholesterol itself. A possible cause for a part, at least, of the excess value may be found in the slight brownish color invariably present in the chloroform solution of extracts prepared by Bloor's method. The alcohol-ether extraction method itself is most excellent, and has been used with good results in gravimetric determinations, as will appear later, but when an aliquot of this is evaporated, no matter how carefully one attempts to avoid heating the residue after it is dry (which Bloor claims to be the cause of the brown color), and even when it is evaporated before a fan at room temperature, there is still a slight brown tinge to the chloroform solution of the residue. This may be, however, only one factor which raises the value, and other things may play even a more important part.

In order to check the error in the digitonin determination, a few analyses were made on Merck's pure cholesterol. The cholesterol was dissolved in alcohol, brought to boiling, and a 1 per cent solution of digitonin in 90 per cent alcohol added, using 25 per cent in excess of the calculated quantity. This proportion of excess of the precipitant has been used in all the work. After precipitation, two procedures are available. In either case several hours are allowed for precipitation to become complete. In this laboratory it has always been placed in the ice box over night. Then, if one follows the original method of Windaus,<sup>2</sup> it is filtered on a Gooch filter, washed with alcohol and ether, dried in the air bath, and weighed. Since the compound is known to be slightly

soluble in alcohol, Fraser and Gardner<sup>6</sup> have modified the Windaus technique by washing with ether followed by boiling water, thus avoiding the use of alcohol in washing, and they use either a Gooch filter or tared filter papers in which the tared paper is washed with ether and water parallel with the paper containing the precipitate. This latter method has several advantages over the Windaus technique. In the first place, once the precipitate becomes firmly packed down on a Gooch filter, subsequent filtration and washing is very slow indeed; moreover, by the use of filter papers, several determinations can be carried out at the same time. The avoidance of alcohol washings probably obviates a slight loss in weight, since the precipitate is somewhat soluble in it. On the other hand, the washing with boiling water is probably a much more effective means of removing excess digitonin from the precipitate. Digitonin in a 1 per cent solution quite readily crystallizes out, and will not again go into solution until the mixture is heated up to about 60°. Therefore, if such a crystallizing out of digitonin should occur in a determination which was subsequently washed with cold alcohol and ether, it would almost certainly not dissolve, and the result would of course be too high. The procedure of Fraser and Gardner has, therefore, been followed in all practical determinations made in this laboratory. The weight of the precipitate is multiplied by the calculated factor 0.2431 to obtain the weight of cholesterol present rather than the approximate factor 0.25 recommended by Windaus to cover loss of the compound in washing.

In four analyses the results were as follows:

Cholesterol.		Remarks.
Taken.	Recovered.	
gm.	gm.	
0.0200	0.0204	Filtered on tared filter papers and washed with ether and boiling water.
0.0200	0.0205	
0.0200	0.0198	Filtered on a Gooch filter and washed with alcohol and ether.
0.0200	0.0201	

Since, then, accurate results are to be obtained by either of the two procedures as long as pure cholesterol is used to begin with,

<sup>6</sup> Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1909, lxxxi, 230.

what is the explanation of the marked discrepancy in analyses of blood? The greatest possibility for error, of course, lies in the preliminary extraction method. A complete extraction cannot be attained by extracting blood directly with an immiscible solvent, because of the formation of emulsions, and also because cholesterol apparently forms some kind of a physical or loose chemical combination with the proteins, from which it cannot be removed by such solvents as ether and chloroform. If the first difficulty is overcome by drying the blood with anhydrous sodium sulfate and grinding, and then extracting with ether in a Soxhlet, still the removal is incomplete.

In the Autenrieth-Funk method the completeness of the extraction depends on the preliminary breaking up of the protein molecules themselves by digesting on a water bath for 2 hours with 25 per cent potassium hydroxide in the proportion of about one part of blood to ten of alkali. This mixture will then give up the cholesterol to either chloroform or ether. Of course in such a process, the esters of cholesterol are saponified, so that it would be useless to extract in this way for the digitonin method, in which the main point is to determine separately the free and combined cholesterol.

If blood is added directly to alcohol, and the precipitate thus obtained extracted with boiling alcohol or by ether, or if blood is run slowly into a mixture of three parts alcohol to one of ether, using three parts of blood to one hundred of solvent, and the whole then brought to a boil on a water bath (Bloor's method) the extraction will be found to be practically complete, as shown by qualitative tests of the protein residue after filtration.

The following experiments will serve to illustrate the foregoing points:

20 cc. portions of dog blood were dried with sodium sulfate and ground. These were then extracted in a Soxhlet apparatus for 1 and 5 days respectively, and the cholesterol was determined in the extracts by the digitonin method. Duplicate portions of 2.5 cc. each were analyzed

	per cent
values, A.....	0.289
B.....	0.287
values, 1 day.....	0.125
5 days.....	0.150

Several specimens of blood were analyzed both colorimetrically using the method of Autenrieth and Funk, and in some instances also that of Bloor, and gravimetrically after precipitating the sample (10 cc.) with alcohol, and extracting the precipitate 24 hours with boiling alcohol in a continuous extraction apparatus, then concentrating the combined alcohol extracts, diluting with water, and extracting with ether.

Following are a few typical analyses:

Source.	Autenrieth-Funk	Bloor	Digitonin
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Dog .....	0 185		0 151
" .....	0 231	0 267	0 169
Horse .....	0 151	0 186	0 100

The protein residues, after extraction with boiling alcohol, were digested for 2 hours with 100 cc. of 25 per cent potassium hydroxide, cooled, and extracted with 50 cc. of chloroform, the chloroform concentrated to about 5 cc., and to this 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid were added. Only a very faint yellowish green color was obtained, showing practically a complete extraction. The color obtained was about equal to that produced by about 0.0001 gm. of cholesterol in the same volume.

The above figures illustrate the lower results obtained by the digitonin method, even when extraction was probably complete. It did not, however, seem quite fair to make comparisons which would leave room for question on this score. Several experiments, of which the following are types, were carried out.

12 cc. of sheep blood were digested for 2 hours with 100 cc. of 25 per cent potassium hydroxide, and the resulting liquid was extracted once with 250 cc. of chloroform and three successive times with 125 cc., shaking vigorously for 5 minutes each time. (This proportion of chloroform to solution and time of shaking are recommended by Autenrieth and Funk.) The combined extracts were shaken out with two small portions of water to decolorize, and then dried over sodium sulfate. After concentrating, the residue was transferred to a 100 cc. volumetric flask, filled to the mark, and 16.7 cc. (= 2 cc. of blood) were removed for colorimetric analysis. The remainder (= 10 cc. of blood) was evaporated to dryness, taken up in alcohol, and precipitated by digitonin. A second portion of blood was analyzed colorimetrically by Bloor's method

	<i>per cent</i>
Colorimetric result..	0 136
Gravimetric " . . .	0 114
Colorimetric " (Bloor)	0 182

The experiment was repeated with 12 gm. of sheep liver, the alkaline mixture being autoclaved 1 hour at 15 pounds' pressure, instead of being boiled for 2 hours.

	per cent
Colorimetric result.....	0.409
Gravimetric " .....	0.374

Dog blood digested as in the case of the sheep blood gave the following figures:

	per cent
Colorimetric result.....	0.182
Gravimetric " .....	0.152
Colorimetric " (Bloor).....	0.216

Bloor states, in accounting for his higher results, that probably a portion of the cholesterol is destroyed by the long continued boiling with strong alkali in the Autenrieth-Funk method. This is a reasonable assumption, and, if true, would equally well explain the lower gravimetric figures. In order to bring about extraction and saponification without the use of such strong alkali the following experiments were done:

Two samples of dog blood were obtained, and 15 cc. portions were extracted by the method of Bloor, using, for each, 500 cc. of a mixture of three parts alcohol and one part ether, and adding the blood slowly to about 400 cc., bringing to a boil, cooling, and making up to the mark in a 500 cc. volumetric flask. The mixture was filtered by suction and the precipitated protein sucked as dry as possible, but not washed. The filtrate was made up to 500 cc. A 5 cc. portion was removed for colorimetric determination. The remaining 495 cc. were in each case evaporated down to 15 to 20 cc., taken up in water, and thoroughly extracted with four portions of ether. The aqueous residue after ether extraction was evaporated to dryness, 25 per cent potassium hydroxide added, and heated for a half hour on the water bath, then extracted with chloroform. The chloroform extract concentrated to about 5 cc. gave only a trace of yellowish color with acetic anhydride and sulfuric acid; hence extraction was complete. The ether extracts were united, concentrated, and made up to 100 cc. A portion equivalent to 2 cc. of the blood was removed for colorimetric analysis, the remaining ether evaporated off, and the residue (= 13 cc. blood) saponified for 2 hours with 50 cc. 0.5 N alcoholic potash under a return condenser. The greater part of the alcohol was then evaporated off, the residue taken up in water, and thoroughly extracted with ether. The aqueous residue again showed no cholesterol when tested as described above. The ether extracts were concentrated, made to 100 cc., a portion equivalent to 2 cc. of blood was removed for colorimetric analysis, a further portion equivalent to 1 cc. removed and discarded, the remaining ether

extract, equivalent to 10 cc. of blood, evaporated to dryness, and the residue taken up in alcohol and precipitated with digitonin.

Determinations were also made on the blood by the Autenrieth-Funk method.

The results were as follows:

	I.	II.
	<i>per cent</i>	<i>per cent</i>
Colorimetric result (Bloor's method, 5 cc. of extract)	0 228	0 293
"    "    ether extract, unsaponified	0 176	0 233
"    "    "    "    saponified	0 174	0 237
Gravimetric    "    ...	0 159	0 208
Colorimetric    "    Autenrieth-Funk method	0 197	0 239

With the exception of the figure obtained by the Autenrieth-Funk analysis of Sample I, which is apparently too high, the figures compare very well with those of the preceding experiments. The striking decrease in the colorimetric value produced only by extracting the concentrated alcohol-ether solution with ether, and thus eliminating some brownish pigment which always is present in the latter, is shown well in both sets of analyses, and there is apparently no further loss after saponification. All of the material, however, which contributes to the green color is evidently not cholesterol, for it cannot be precipitated by digitonin.

There was still the chance that the 2 hours' saponification with alcoholic potash so altered some of the cholesterol that, while it still gave the color reaction it failed to precipitate with digitonin.

To see if this were the case, two samples of 10 cc. each of horse blood were extracted by Bloor's method, and to the alcohol used in one case 0.02 gm. of pure cholesterol was added. These two extracts were saponified and precipitated as above, the resulting amounts of cholesterol obtained being respectively 0.0076 and 0.0274 gm. There was consequently 0.0198 gm. of the cholesterol recovered in spite of the 2 hours' saponification. This made it pretty certain that there was little change in the cholesterol by the alkali, for by mass action more cholesterol should have been changed in the second case than in the first, if a change took place, and a greater loss would have resulted. But to avoid any error on this point, the effect of saponification with 0.5 N alcoholic potash was tried on pure cholesterol. Three portions of 0.0100 gm. of cholesterol in 10 cc. of alcohol were pre-

pared. One portion was immediately precipitated with digitonin. The second was saponified for 1 hour, and the third for 2 hours, then extracted and precipitated. The amounts recovered were respectively 0.00938, 0.00948, and 0.00943 gm., so that there was practically no loss after 2 hours' saponification.

In a number of determinations the ether washings from the digitonin-cholesterol precipitate were extracted once or twice with water to remove the excess digitonin, evaporated to dryness, taken up in 5 cc. of chloroform and acetic anhydride, and sulfuric acid was added. After a short time an unmistakable olive green color always appeared, having, however, a brownish cast. This was equal in intensity perhaps to that which would be produced by 0.002 gm. of cholesterol, and, since the precipitate of cholesterol in the average experiment was usually about 0.015 gm., it amounts to considerably more than 10 per cent of the total color which could have been produced by the material before precipitation. The green color produced by these filtrates faded out rather more quickly than the cholesterol color, and left a yellowish brown solution. In one case, where the residue was treated with glacial acetic acid and a few drops of concentrated sulfuric acid, a slightly reddish color was produced (oxycholesterol reaction?—see below).

#### DISCUSSION.

It is apparent from the foregoing that the values obtained by the colorimetric method represent some additional substance or substances beside cholesterol itself. That substances closely related to cholesterol exist in the blood normally is shown by the work of Lifschütz.<sup>7</sup> So called "oxycholesterol," a substance which may be prepared artificially from cholesterol by oxidation with potassium permanganate, ferric chloride, or benzoyl peroxide in glacial acetic acid solution, and characterized chemically by the production of a reddish violet color passing into green, with definite spectrum lines upon the addition of sulfuric acid to its acetic acid solution, has been isolated by him from the blood, and a method worked out for its quantitative determination.

<sup>7</sup> Lifschütz, J., *Z. physiol. Chem.*, 1906-07, 1, 437; 1907, liii, 140; 1908, lviii, 175.

There is some question as to the properties of these oxidation products, of which there are two or three which can be recognized spectroscopically. Lifschütz<sup>8</sup> states that they will give the Liebermann-Burchard color reaction, and also that they will precipitate with digitonin from alcohol solutions, while Schreiber<sup>9</sup> denies the latter point. Since much of this oxysterol work remains as yet unconfirmed it is scarcely desirable to speculate about the exact part which is played by these derivatives in the results obtained in "cholesterol" determinations. Certain it is that they, or some related substances present in the blood, add to the green color of the Liebermann-Burchard test, but fail to precipitate with digitonin. It is quite possible that there may be a partial precipitation of some of this material by the digitonin, and that the gravimetric results themselves are somewhat in excess of the actual amount of true cholesterol present. No attempt has been made to determine whether such a condition exists or not. In one experiment it was shown that there was no wide divergence from the theoretical formula for the precipitate by boiling a sample weighing 0.0111 gm., which had been obtained from dog blood, with 0.5 N alcoholic potash for 2 hours, which gradually dissociates the compound and destroys the digitonin. After a thorough ether extraction of the residue, there could be recognized colorimetrically 0.0026 gm. of cholesterol, while the amount present theoretically was 0.0027 gm. Of course the presence of any oxysterol-digitonin compound is not disproved by such an experiment, since the formulas would be closely related, cholesterol being  $C_{27}H_{44}O$  and oxysterol  $C_{27}H_{44}O_2$ . For the present, however, it seems warranted to look upon the digitonin determinations of cholesterol in blood as being very nearly correct values when care is taken to insure complete extraction. Values obtained by colorimetric methods should be considered as representing cholesterol plus some more or less closely related substance or substances, very likely of the nature of oxidation products.

<sup>8</sup> Lifschütz, *Munch. med. Woch.*, 1913, lx, 1549, 2346.

<sup>9</sup> Schreiber, E., *Munch. med. Woch.*, 1913, lx, 2001.



## CONCLUSIONS.

1. When applied to solutions of pure cholesterol either the colorimetric or the digitonin method of determination is accurate.

2. Ether extraction of dried blood, even when prolonged, is not complete, and recourse must be had to hot alcohol or alcohol and ether.

3. Colorimetric analyses of blood give results too high for true cholesterol, because they include other ether- and chloroform-soluble substances, whereas digitonin determinations are more nearly correct.

# THE INFLUENCE OF AUTOLYSIS UPON CHOLESTEROL ESTERS.

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It has been shown<sup>1</sup> that cholesterol is absorbed into the animal body from the intestinal tract by way of the thoracic duct. No matter whether it is fed in the free condition or in the form of esters of the higher fatty acids, it is always found to have been converted to a mixture of about one part free to three or four parts ester by the time it reaches the chyle.

There are probably two quite distinct functions served by these two forms. Nothing can be said of the use of the esters of cholesterol, while in the free condition it is known to act at least *in vivo* as a neutralizing substance to a number of different poisons, such as saponin,<sup>2</sup> tetanolysin,<sup>3</sup> a hemolytic agent of the pneumococcus,<sup>4</sup> and also conceivably, poisons of metabolic origin. It seems desirable then, to study any further interchange between the two forms which may take place in the body.

A review of results reported by many different observers shows clearly that the total cholesterol of the blood serum of practically all species of animals reported upon (exclusive of human serum under pathological conditions) is always made up of both free and combined fractions, 25 to 35 per cent of the total being free. It will be noted that this is the proportion in which the cholesterol appears in the chyle after its absorption. When analyses of the red or white blood cells or of practically any

<sup>1</sup> Mueller, J. H., *J. Biol. Chem.*, 1915, xxii, 1.

<sup>2</sup> Ransom, F., *Deutsch. med. Woch.*, 1901, xxvii, 194.

<sup>3</sup> Noguchi, H., *Univ. Pennsylvania Med. Bull.*, 1902, xv, 327.

<sup>4</sup> Cole, R., *J. Exp. Med.*, 1914, xx, 346.

normal tissue, are made, the results are entirely different. In the red cells,<sup>5</sup> for example, there is probably no combined cholesterol; very little is found in the white cells, and the same is true for most other tissues. The brain contains practically no esters;<sup>6</sup> the kidney,<sup>7</sup> heart,<sup>8</sup> and liver contain varying proportions, from one-tenth to one-half of the total.

There are possibly two explanations for such a condition. Either the body cells are able by means of enzymes to split the esters of the blood plasma, and absorb the free cholesterol, or they absorb the free cholesterol selectively, and the esters are destroyed or altered in some way other than by simple hydrolysis. The first supposition seems the more reasonable, and experiments directed at the recognition of such enzymes have been carried out. It may be stated here that the results have been negative, but for reasons which will appear later it seemed worth while to present the material briefly at this stage.

The attempt has been made to show, by means of autolysis of blood, liver, and mixtures of the two, that there occurred hydrolysis of the esters of cholesterol which normally exist in such materials. This form of experiment and the selection of this material was suggested by two reports in the literature, and it was hoped to make these the basis for more extended work.

Schultz<sup>9</sup> autolyzed human, horse, and dog blood, and also liver and mixtures of blood and liver in the presence of sodium fluoride and thymol or chloroform as preservatives. He was unable to get any evidence of hydrolysis in blood alone, but in horse liver alone and in mixtures of horse, and, to a less extent, of sheep blood and liver, marked hydrolysis took place. Cytronberg,<sup>10</sup> in the same laboratory, reported positive results on horse and dog blood alone, and ascribed Schultz's failure to insufficient familiarity with the digitonin method of analysis. This work has

<sup>5</sup> Wacker, L., and Hueck, W., *Arch. exp. Path. u. Pharm.*, 1913, lxvii, 416.

<sup>6</sup> Lapworth, A., *J. Path. and Bact.*, 1911, xv, 254.

<sup>7</sup> Windaus, A., *Z. physiol. Chem.*, 1910, lxx, 110.

<sup>8</sup> Hess-Thaysen, T. E., *Habilitationsschrift*, Copenhagen, 1913; abstracted in *Centr. Biochem. u. Biophysik.*, 1913-14, xvi, 85.

<sup>9</sup> Schultz, J. H., *Biochem. Z.*, 1912, xlii, 255.

<sup>10</sup> Cytronberg, S., *Biochem. Z.*, 1912, xlv, 281.

remained unconfirmed, except for the statement of Gardner and Lander<sup>11</sup> that in an autolyzed eat liver, no cholesterol esters could be found.

There are certain peculiarities about the figures quoted by Schultz and by Cytronberg, which may be mentioned briefly. Both workers analyzed equal quantities of blood, usually 200 cc. before and after autolysis. Corper<sup>12</sup> and Thiele<sup>13</sup> have shown that total cholesterol is not decreased on autolysis of tissues. Schultz's figures, however, nearly always show a loss of a third to a half of the total cholesterol after 4 days' autolysis, and in one case of blood and liver, a loss of two-thirds. This loss is not mentioned in his discussion of his results. It is hard to explain so much loss, even by incomplete extraction, but no other explanation is apparent. The figures of Cytronberg do not show this decrease, but his analyses of fresh blood are most peculiar in that they show only about 30 per cent of the total cholesterol to be in the free condition. From analyses of whole blood, together with analyses of plasma, he calculates in two instances that the red cells contain about 60 per cent of their total cholesterol as esters. Most analyses available for reference place the free cholesterol in whole blood at between 60 and 70 per cent of the total. The figures given below for fresh blood illustrate this well. Furthermore, it is now generally recognized that the red cells contain cholesterol esters only in traces, if at all. Apparently, then, the analytical results in both papers are open to criticism.

#### EXPERIMENTAL.

All blood and tissue used were obtained as fresh as possible, and always used within an hour. Blood was diluted with an equal volume of water before autolysis. In mixtures of liver and blood, the finely ground liver was mixed with an equal weight of blood, and one volume of water was added. For preservatives sodium fluoride, chloroform, trieresol, and toluene were used, in various experiments. It may be said that in some of the experiments, in which liver was autolyzed, there was apparently so

<sup>11</sup> Gardner, J. A., and Lander, P. E., *Biochem. J.*, 1913, vii, 576.

<sup>12</sup> Corper, H. J., *J. Biol. Chem.*, 1912, xi, 44.

<sup>13</sup> Thiele, F. H., *Biochem. J.*, 1913, vii, 275.

much putrefaction, in spite of a reasonable amount of preservative, that positive results would have been questioned, even if obtained. The effort was made in all the work to use the minimum amount of antiseptic effective to prevent putrefaction, in order to avoid enzyme inhibition as far as possible. In most cases extraction has been carried out with boiling alcohol, but in a few cases ether has been used after drying with sodium sulfate. Such results are probably too low, but, even so, marked hydrolysis should have been apparent. The alcoholic extracts are concentrated, diluted with water, and extracted by ether. The ether extract is divided into equal parts, one-half saponified with alcoholic potash for 2 hours, and again extracted. After evaporation of the ether from both fractions, each is taken up in 10 cc. of alcohol and precipitated by digitonin. The weight of the digitonin-cholesteride multiplied by 0.2431 gives in one case the free cholesterol and in the other the total.

The figures in the column headed "ratio" are obtained by dividing the percentage of total cholesterol in the material by the percentage of free.

#### DISCUSSION.

Slight variations in the results obtained for total cholesterol before and after autolysis are attributable to experimental error in the method. In none of the experiments is there any such evidence of hydrolysis as has been reported by Schultz and Cytronberg. In one experiment quoted by the latter the ratio of free to total cholesterol rose from 36.6 to 91.7 during 4 days' autolysis, and other figures were almost as striking. It can only be concluded from the figures here quoted, that a careful repetition of their work has entirely failed to confirm their results.

It is hoped that, later on, further work by different methods may throw some light on the main question of whether or not the tissues during life can hydrolyze the esters of cholesterol.

Material	Amount	Total	Free	Ratio	Remarks
	cc	per cent	per cent	per cent	
Ox blood, fresh	20	0 120	0 081	67 5	CHCl <sub>3</sub> and NaF
4 days' autolysis	20	0 108	0 084	77 7	
Ox blood, fresh	20	0 099	0 050	50 51	CHCl <sub>3</sub> and NaF
4 days' autolysis	20	0 094	0 047	50 00	
Sheep blood fresh	20	0 051	0 031	61 54	
4 days' autolysis	20	0 056	0 032	56 90	CHCl <sub>3</sub> and NaF.
4 " "	20	0 057	0 033	57 63	NaF
Dog blood, fresh	20	Lost	Lost		
4 days' autolysis	20	0 123	0 081	65 68	CHCl <sub>3</sub> and NaF
4 " "	20	0 131	0 082	62 76	NaF
Fresh	20	0 169	0 102	60 11	Same dog bled 3 days later
Horse blood, fresh	20	0 100	0 065	65 00	
4 days' autolysis	20	0 099	0 060	60 54	CHCl <sub>3</sub> and NaF
	gm				
Sheep blood and liver, fresh	25	0 242	0 125	51 82	Measurement of amount taken only approximate
4 days' autolysis	25	0 300	0 162	53 93	NaF, trieresol, and CHCl <sub>3</sub>
Horse blood and liver, equal parts, fresh	25	0 123	0 107	87 01	
4 days' autolysis	25	0 116	0 105	90 65	NaF and CHCl <sub>3</sub>
	cc				
Horse blood citrated, fresh	20	0 108	0 087	80 55	Ether extraction Note increase in total amount extracted after autolysis
1 month's autolysis	20	0 132	0 082	62 15	Trieresol
Horse blood, defibrinated, fresh	20	0 114	0 092	80 70	Trieresol
1 month's autolysis	20	0 159	0 102	64 77	"
		gm	gm		
Horse blood and liver, equal parts, fresh		0 1086	0 1033	95 1	Ether extraction
4 day's autolysis		0 0399	0 0036	90 9	Trieresol and toluene.



## STUDIES ON BLOOD SERUM.

### I. THE DETERMINATION OF NON-COLLOIDAL NITROGEN.

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#### INTRODUCTION.

In recent years, knowledge regarding the amount of non-colloidal nitrogen present in the blood has become of more and more importance to the clinician and the biochemist. In connection with our work on the Abderhalden serum reaction, it appeared to us that a quantitative determination of the non-colloidal nitrogen formed by the tryptic activity of the serum would be of more value than a qualitative test on the same by the ninhydrin reaction.

Folin and Denis<sup>1</sup> proposed a method for the determination of the non-protein nitrogen of the blood in which methyl alcohol is used as the precipitant, and the filtrate is subsequently treated with zinc chloride for the removal of some colloidal nitrogenous substances that are not precipitated by the methyl alcohol under the conditions of the experiment. Believing that aluminium hydroxide cream<sup>2</sup> had certain advantages over methyl alcohol as a precipitant for the colloids of the serum, we selected it as our reagent for this purpose. ♦

#### EXPERIMENTAL.

In this study, we compared the results obtained by the use of the method described by these investigators with those obtained by the use of the aluminium hydroxide cream method. The

<sup>1</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

<sup>2</sup> Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, xxxv, 820.  
Tracy, G., and Welker, W. H., *J. Biol. Chem.*, 1915, xxii, 55.



latter method was carried out as follows: 5 cc. of serum were accurately measured into a 250 cc. volumetric flask. 75 cc. of aluminium cream were then transferred to the flask and the contents were mixed by shaking, and following this were diluted to the mark with distilled water. A thorough mixing of the contents was followed by filtration. The total nitrogen was determined in 125 cc. of the filtrate by the Kjeldahl method. In all cases, the filtrates from the serum treated with aluminium cream failed to show any protein by Heller's ring test or by the coagulation test. A blank determination was run on the aluminium cream and this result was subtracted from that obtained with the aluminium-treated serum.

The aluminium cream method is simpler in technique than that suggested by Folin and Denis, since only one filtration is required. There is less danger of error through evaporation of the solution. Our results, which are averages of two or more closely agreeing determinations, show the recovery of non-colloidal nitrogen when added to serum. The substances first studied were urea, creatine, glycocoll, and leucine (Table I).

TABLE I

Method	Serum added cc	Urea cc	N •		Glycocoll		Leucine		Creatine 1		Creatine 2	
			mg	cc	mg	cc	mg	cc	mg	cc	mg	cc
Kjeldahl		2.5	14.07	2.5	2.24	2.5	0.70	2.5	1.75	2.5	1.51	
Aluminium (Kjeldahl)		5	14.07	5	2.24	5	0.70	5	1.68	5	1.36	
“ “	5	—	0.98	—	1.12	—	1.12	—	0.91	—	0.84	
Alcohol “	5	—	1.12	—	1.26	—	1.26	—	0.98	—	0.98	
Aluminium “	5	5	14.95	5	3.29	5	1.75	5	2.24	5	2.02	
Alcohol “	5	5	15.18	5	3.42	5	1.89	5	2.17	5	2.10	
Aluminium (by difference)	5		13.97	5	2.17	5	0.63	5	1.33	5	1.18	
Alcohol “ “	5		14.06	5	2.16	5	0.63	5	1.19	5	1.12	

\* These results, in all cases except where the Kjeldahl method was applied directly, are equivalent to one-half the volume of the samples indicated, since the determinations were carried out on one-half the volume to which the samples were originally made up.

In a subsequent series of experiments, in order to make our conditions approximate those in the Abderhalden test, we used solutions which we have designated Dialysates 1 and 2. The-

solutions were obtained by subjecting dog serum to tryptic digestion at 40°C. and dialyzing the resultant mixture in parchment paper against distilled water. The dialysates were free from the more complex protein compounds as evidenced by negative Heller's ring and coagulation tests. Neither of the dialysates showed a precipitate with picric acid, but they gave a faint biuret and a very intense ninhydrin test. These dialysates represented a mixture of protein cleavage products similar to those obtained in the Abderhalden tests. The results are shown in Table II.

TABLE II

Method.	Serum	Dialysate	Nitrogen *	
			1	2
	cc	cc	mg	mg
Kjeldahl....		2 5	0 84	1 33
Aluminium (Kjeldahl)		5	0 74	1 12
“ “	5	—	1 33	0 91
Alcohol “	5	—	1 06	0 98
Aluminium “	5	5	2 17	2 10
Alcohol “	5	5	1 82	2 17
Aluminium (by difference)		5	0 84	1 19
Alcohol “ “		5	0 76	1 19

\* These results, in all cases except where the Kjeldahl method was applied directly, are equivalent to one-half the volume of the samples indicated, since the determinations were carried out on one-half the volume to which the samples were originally made up.

## DISCUSSION.

The results show that the nitrogen of urea, glycocoll, and leucine can be recovered to a very large extent, and that creatine nitrogen can be recovered only in part by this method. In the experiments on the solution of the non-colloidal products of the tryptic digestion of the serum proteins there is also a failure to recover all the added nitrogen. The results obtained by the use of this method show that not all the non-colloidal nitrogen is determined. They follow closely, however, those obtained by the use of the Folin and Denis method.

## CONCLUSIONS.

Aluminium cream is a satisfactory reagent for the removal of the colloidal nitrogen from blood serum. Its use simplifies the determination of the non-colloidal nitrogen of the serum. The liability to experimental error is less than in the method proposed by Folin and Denis.

# THE MECHANISM OF THE SPARING ACTION OF CARBOHYDRATES ON PROTEIN METABOLISM.

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(Received for publication, May 4, 1916.)

Voit,<sup>1</sup> Rubner,<sup>2</sup> Benedict,<sup>3</sup> Sivén,<sup>4</sup> Landergren,<sup>5</sup> and others have repeatedly demonstrated that ingested carbohydrates exert a sparing effect on the metabolism of body protein. This sparing action is considerably more marked with carbohydrates than with fats. It is possible with the ingestion of large amounts of carbohydrates to reduce the nitrogen output in man from the starvation figures of 8 or 10 gm. to as low as 3 gm. If the carbohydrates are omitted from the diet or even replaced by fat, the nitrogen output again increases to more than double this minimum. Loewi,<sup>6</sup> Luthje,<sup>7</sup> Lesser,<sup>8</sup> and others further showed that carbohydrates are necessary for the utilization of the split products of protein. Without carbohydrates there is no retention of nitrogen on feeding mixtures of the abiuret split products. In explanation of this, Luthje thought that an amino sugar was formed. There is, however, no experimental evidence to support this view. According to Landergren, since under physiological conditions fats cannot supply this want, the proteins are broken down to yield the sugar

<sup>1</sup> Voit, C., Hermann's Handb. Physiologie, Leipsie, 1881, 140.

<sup>2</sup> Rubner, M., Die Gesetze des Energieverbrauchs bei der Ernährung, Vienna, 1902.

<sup>3</sup> Benedict, F. G., *Carnegie Institution of Washington, Publication No. 77*, 1907.

<sup>4</sup> Sivén, V. O., *Skand. Arch. Physiol.*, 1900, x, 91; 1901, xi, 308.

<sup>5</sup> Landergren, E., *Skand. Arch. Physiol.*, 1903, xiv, 112.

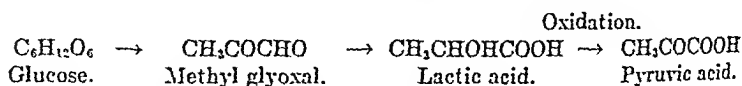
<sup>6</sup> Loewi, O., *Arch. exp. Path. u. Pharm.*, 1902, xlviii, 303.

<sup>7</sup> Luthje, H., *Arch. ges. Physiol.*, 1906, cxiii, 547.

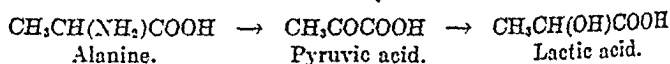
<sup>8</sup> Lesser, E. J., *Z. Biol.*, 1904, xlv, 497.

which is lacking. This results in an increase in the nitrogen output. Cathcart<sup>9</sup> suggested that carbohydrate is essential to protein synthesis, and this view is held also by Janney.<sup>10</sup>

Sugar, except when stored as glycogen, is dissociated in a definite way in the body into simpler molecules. An idea of the relationship of glucose to the dissociation products may be gathered from the following scheme:



Of special interest in this connection is the fact demonstrated by Otto Neubauer that keto and hydroxy acids identical with those arising in the course of sugar catabolism may result from the dissociation of proteins in the body. For example, alanine is deaminized to form pyruvic acid, which by reduction gives rise to lactic acid according to the following scheme:



It has been shown by a number of workers that the above reactions are reversible. For example, lactic acid and pyruvic acid may give rise to glucose in a phlorhizinized animal. The increased glycosuria in phlorhizinized animals following ingestion of proteins is a further evidence of the reversal of this reaction. That the second of these reactions is reversible, namely, the synthesis of hydroxy and keto acids into amino-acids, has been demonstrated by Knoop<sup>11</sup> as well as by Embden<sup>12</sup> and others. They have shown that on perfusing the isolated liver with lactic or pyruvic acid, nitrogen can be added on and alanine formed. If this process of retaining nitrogen by dissociation products of sugar to form new amino-acids, and hence proteins, occurs on a large scale in the body, it will explain why ingestion of carbohydrates

<sup>9</sup> Cathcart, E. P., *The Physiology of Protein Metabolism*, London, 1912, 121.

<sup>10</sup> Janney, N. W., *J. Biol. Chem.*, 1916, xxiv, p. xxx.

<sup>11</sup> Knoop, F., *Z. physiol. Chem.*, 1910, lxxvii, 489.

<sup>12</sup> Embden, G., and Schmitz, E., *Biochem. Z.*, 1910, xxix, 423.

spares body protein. To test this point, the sparing effect on the nitrogen output of ingesting lactic and pyruvic acids as compared with the sparing action of equivalent amounts of undissociated carbohydrates was undertaken in these experiments.

#### EXPERIMENTAL.

Large healthy dogs were used for the experiments. They were allowed to fast for 5 or 6 days. When the nitrogen output became constant, lactic acid, pyruvic acid, or cane sugar was given by stomach tube. The lactic acid was partly neutralized by calcium hydroxide; pyruvic acid was neutralized with sodium hydroxide. For comparative purposes the sparing action of cane sugar was first determined for each dog. The urine was collected every 24 hours by catheterization. During fasting the dogs received daily a liter of water, and the carbohydrates and acids were administered in an equivalent dilution, so that the daily quantity of urine was kept constant. The results of the experiments are tabulated in Tables I to III.

TABLE I

*Experiment 1.*—A healthy bitch weighing 19.5 kg. Cane sugar was given on the 7th day; lactic acid partly neutralized with calcium hydroxide was given by stomach tube on the 10th day.

Day	Food	N in the urine	Remarks
		gm	
5	Fasting	2.86	
6	"	2.92	
7	95 gm. cane sugar	1.66	
8	Fasting..	2.56	
9	"	2.84	
10	100 gm. lactic acid.	1.82	3.32 gm. of <i>l</i> -lactic acid were recovered in the urine.
11	Fasting	2.48	
12	"	3.03	

TABLE II.

*The Effect of Lactic Acid on the N Output of the Dog.*

*Experiment 2.*—Dog weighing 18.6 kg. In this experiment doses of cane sugar and lactic acid were given over 3 days in amounts of 80 and 80 gm. respectively.

Day	Food	N in the urine.  gm.	Remarks.
5	Fasting .....	3.35	
6	" .....	3.49	
7	76 gm. cane sugar...	2.25	
8	76 " " " ...	1.74	
9	76 " " " ...	1.27	
10	Fasting ....	1.44	
11	" .....	2.22	
12	" .....	3.19	
13	" .....	3 40	
14	80 gm. lactic acid...	2.68	Traces of lactic acid found in the urine.
15	80 " " " ...	2 19	" " " " " " " "
16	80 " " " .	1 65	" " " " " " " "
17	Fasting . . .	2 03	
18	" .. .	2 55	

TABLE III.

*Experiment 3.*—Same dog as in Experiment 2. Pyruvic acid neutralized with sodium hydroxide was given in doses of 80 gm. per day for 3 days. The pyruvic acid was prepared in the laboratory by the distillation of tartaric acid and potassium hydrogen sulfate. It was freshly distilled before use

Day	Food	N in the urine  gm	Remarks
6	Fasting .	3 52	
7	"	3 41	
8	80 gm. pyruvic acid	2 88	
9	80 " " "	2 44	
10	80 " " "	2 18	Traces of l-lactic acid were detected in the urine.
11	Fasting .	2 56	
12	" .. .	2 85	
13	" ...	3 36	

Following the ingestion of cane sugar there was the usual reduction of the nitrogen output. In these experiments this reduction was down to approximately 53 per cent of the fasting nitrogen when 100 gm. of carbohydrates were given on 1 day only; following the administration of 76 gm. a day for 3 consecutive days, the nitrogen output was reduced to about 37 per cent of the fasting nitrogen. These figures serve as a basis of comparison for the sparing action of equivalent amounts of lactic and pyruvic acids. In Experiment 1 where 100 gm. of lactic acid were given, the nitrogen output was reduced from 2.84 to 1.82 gm., or 64 per cent of the fasting nitrogen. 3.32 gm. of *l*-lactic acid were recovered from the urine. It is apparent that not quite all of the lactic acid had been utilized, but the reduction in the nitrogen output to 64 per cent as compared with 53 per cent for an equivalent amount of cane sugar indicates that lactic acid spares protein to practically the same extent as do carbohydrates. In Experiment 2 lactic acid was given in amounts of 80 gm. on 3 consecutive days. The daily nitrogen output was reduced from 3.4 gm. to 1.65 gm. on the 3rd day, or 48 per cent of the fasting nitrogen as compared with a reduction to 37 per cent following equivalent amounts of cane sugar. There were traces of lactic acid found in the urine, so that it is likely that had all the lactic acid been utilized reduction in nitrogen output would have been fully as great as that following carbohydrates. In Experiment 3 the effect of ingesting pyruvic acid was studied. 80 gm. doses per day were given for 3 days. The nitrogen output fell from 3.41 to 2.18 gm. on the 3rd day, or 64 per cent as compared with 37 per cent following carbohydrates, and 48 per cent following lactic acid. There were no toxic symptoms apparent following administration of these amounts of pyruvic acid in the form of the sodium salt. A trace of *l*-lactic acid was detected in the urine on the 3rd day following the administration.

#### DISCUSSION OF RESULTS.

Lactic acid exerts practically the same sparing action on protein metabolism as carbohydrates. Following pyruvic acid the sparing action is very distinct but less marked than that following lactic acid and carbohydrates. The dissociation of glucose in



the body is by way of lactic acid as one of the chief intermediate steps. Lactic acid can be oxidized in the body to pyruvic acid. As pointed out in the introductory remarks, it has been shown that lactic acid as well as pyruvic acid can add on ammonia nitrogen to form alanine. When this process is operative, nitrogen arising from catabolism of body proteins instead of being excreted is utilized to synthesize new protein. It is true that the above reactions are reversible, as has been shown in phlorhizinized animals; but the normal catabolism of glucose is in the direction indicated, giving rise to simpler molecules, which in turn may be in part combined with nitrogen to synthesize protein. That this fixing of catabolized nitrogen by the dissociation products of glucose to form new proteins is the true mechanism of the sparing effect of feeding carbohydrates on the nitrogen output gains further support in the experiments here reported.

## THE DISTRIBUTION OF THE LIPOIDS ("FAT") IN HUMAN BLOOD.

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(Received for publication, May 31, 1916.)

Those constituents of the blood which are usually included in the term "blood fat" are (1) the true fats—glycerides of the fatty acids; (2) the phosphatides—lecithin, cephalin, etc.—ordinarily termed "lecithin," and (3) cholesterol with its fatty acid esters. At first these substances were grouped together because of their similar solubilities in the fat solvents, but evidence has been accumulating (see below) which makes it probable that the grouping is a natural one based on their common connection with fat metabolism. For this reason it is increasingly desirable to have a general term which shall include all these substances and also others which are concerned in fat metabolism. Three terms have been used for this purpose. The term "*lipin*" suggested by Gies,<sup>1</sup> although good, has not found general acceptance possibly because the ending *-in* is still associated in most minds with basic properties. Leathes<sup>2</sup> uses the term "*fats*" in the general sense to include all substances connected with fat metabolism. This term has the advantage of quite general application in this sense and would be useful if it were not for the fact that the best known member of the group is also universally known by that name. The third term, "*lipoid*," first used by Overton, has two meanings in the literature—the original meaning given it by Overton including essentially all substances having the solubilities of the fats, and a later restricted meaning including only such substances as "lecithin," cholesterol, etc. At present it is coming into general use in France<sup>3</sup> and to a certain extent in Germany<sup>4</sup> as a general term to include all substances connected with fat metabolism. On the whole, it seems to be the

best of the three terms for the purpose. Its root meaning is broad enough to allow its use in this sense and there is an advantage in the use of a familiar term rather than a new one. It will therefore be used in the present work in the above sense to include all substances connected with fat metabolism; i.e., the fatty acids, their naturally occurring compounds, and also substances like cholesterol which occur naturally in combination with the fatty acids.

As might be expected from the importance of fat in general metabolism a considerable volume of literature has accumulated on the lipoids of the blood, but the published results, although in general agreement, vary greatly in the quantitative details. The reasons for the variations in reported values are, first, the widely different and frequently inadequate methods employed; second, the failure in many instances to take into account the nutritional condition of the subject at the time the blood was taken. Practically the only time when the blood is free from the influence of ingested or mobilized fat and when therefore the fat values are constant enough to be used as a basis for comparison, is the post-absorptive condition—8 to 16 hours after the last meal. A third reason is the probable presence in the blood of enzymes<sup>5, 66</sup> which may alter or destroy the lipoids if the blood is allowed to stand for any considerable length of time after being drawn. For these reasons only a general review of the subject is thought to be of value at the present time.

*Fat, Glycerides of the Fatty Acids, Neutral Fat.*—Very little is known regarding the amount of fat in the blood at any time. The methods employed for the determination of lipoids in blood are mainly saponification methods giving values only for total fatty acids, and when suitable methods have been employed the analyses are rarely complete so that the amount of fat could not be determined. Letsche's<sup>6</sup> analyses lead him to the conclusion that the amount of fat present in normal fasting blood is small and may be entirely absent. Klemperer<sup>7</sup> comes to a similar conclusion regarding the blood in certain cases of diabetes.

During fat absorption the fat of the blood is increased as a result of the inflow of chyle fat which consists almost entirely of glycerides. Müller<sup>22</sup> finds the fats increased in nephritis, and Klemperer<sup>7</sup> in the lipemia of diabetes.

*Total Fatty Acids.*—More information is available regarding the total fatty acids of the blood, since most of the methods for determining blood "fat" are saponification methods and involve the separation of the fat as fatty acids; but the values reported for both normal and pathological conditions vary extremely among themselves for the reasons mentioned above.

Very little is known regarding changes in normal human beings in the total fatty acids of the blood other than in the well known alimentary lipemia. In normal animals the total fatty acids have been shown to increase as the result of fasting,<sup>8-12</sup> narcotics,<sup>10, 12, 13</sup> and exercise.<sup>14</sup> The increases in fasting and narcosis are not constant but seem to depend on the nutritional condition of the animal. Animals whose blood lipid level is ordinarily not affected by fasting or narcosis show marked increases in both conditions after a period of stuffing with fat food. Apparently in these animals some of the fat is so loosely stored that it is thrown into the blood as the result of a stimulus which does not affect the animal in his ordinary condition. Rosenfeld<sup>72</sup> many years ago observed that the fat mobilization ordinarily produced by phosphorus poisoning in dogs does not take place in starving animals.

Fat injected into the circulation in suspension disappears from the blood at a rate which appears to depend on the fineness of the suspension. Injected as a colloidal suspension it disappears in about  $\frac{1}{2}$  hour,<sup>15</sup> as casein emulsion (particles 2 to 5  $\mu$  in diameter) in less than 5 minutes,<sup>12</sup> as egg yolk, in 6 or 7 hours,<sup>12</sup> as homogenized fat (consisting of particles 0.3 to 1  $\mu$ ) in about 7 hours.<sup>16</sup>

In pathological conditions in human beings considerable work has been done, particularly in diabetes mellitus. Diabetes is the only common disease in which the blood lipoids are excessively increased and consequently it was the first disease in which attention was directed to these constituents. The milky serum from the blood in many cases of diabetic coma, from which frequently a "cream" arose on standing, suggested fat at once to the observant at the time when blood letting was a common practice, and the earliest information on blood lipoids dates from that time.<sup>17</sup> Our present knowledge regarding the total fatty acids of the blood in diabetes may be summed up as follows.

In diabetics free from acidosis they are not much, if any, higher than in the normal. Acidosis is generally accompanied by increased total fatty acids in the blood but not by any means invariably.<sup>7, 17-21</sup> In coma the values may be very high; 26 per cent is reported by Klemperer,<sup>21</sup> and 15 to 20 per cent appears to be fairly common. On the contrary, lipemia is frequently absent in coma.<sup>21</sup> It seems probable that while the coma and the lipemia may be the result of the same set of conditions the lipemia may develop so gradually that coma may ensue before the lipemia has reached notable proportions. It is questionable whether the increased fat is due to cellular breakdown, as is frequently stated (because of the increase of "lecithin" and other cellular constituents). It seems more reasonable to suppose that the increase represents the accumulation of mobilized or ingested lipoids which the organism is no longer able to utilize.

The "total fatty acids" of the blood have been found to be increased in nephritis,<sup>18, 22</sup> in pneumonia,<sup>18</sup> in pregnancy,<sup>23</sup> and in experimental anemia in animals.<sup>24</sup>

*Phosphatides ("Lecithin")—Lecithin, Cephalin, Etc.*—The "lecithin" of the blood has been reported to increase in normal animals during the absorption of lecithin<sup>25</sup> and of fat.<sup>26, 27</sup> In pathological conditions in human beings increases in blood "lecithin" have been reported in diabetic lipemia,<sup>21, 28, 29</sup> although here as in the case of the fat the increases do not appear to be constant or characteristic since other cases have been reported,<sup>30, 31</sup> even with coma, where the changes were slight. Other pathological conditions in which abnormal values for "lecithin" have been reported are: nephritis, both high<sup>22, 32</sup> and low values;<sup>25, 32</sup> leukemia, high values<sup>32</sup> (in corpuscles only—plasma normal); syphilis, high values (serum);<sup>28, 33</sup> in cachexia (carcinoma) low values.<sup>34</sup> In experimental conditions in animals increases in blood "lecithin" have been reported in the anemia produced by continued bleeding<sup>24</sup> and in depancreatized dogs.<sup>35</sup>

The divergence in the results of lecithin determinations in blood, particularly the low values, may be due to one or both of two causes: (1) destruction of lecithin in the blood by standing, due to the action of an esterase in the corpuscles;<sup>5</sup> or (2) destruction during extraction. Many of the blood samples reported on were obtained post mortem and therefore after standing several

hours; also the methods used for extraction in most cases would undoubtedly result in a partial hydrolysis.

*Cholesterol*.—Cholesterol is a relatively well defined and stable chemical substance, is readily extracted from the blood and purified, and is readily acted upon by a number of reagents producing precipitates or colors which may be used for its quantitative determination. For these reasons it has been the subject of a good deal of investigation in recent years. Also by reason of its stability the values reported for cholesterol in blood are much more comparable than those for "lecithin," although here again the lower values may be regarded as doubtful, either because of incomplete extraction or because of the action of the strong alkali used for hydrolysis preparatory to extraction in most of the methods, since cholesterol is not entirely stable in the presence of strong alkalies.<sup>36</sup> Cholesterol occurs in the blood in two forms; "free" in the corpuscles and to some extent in the plasma, and as cholesterol esters in the plasma alone. The relation of free to bound cholesterol in the plasma is variously given.<sup>34</sup> One part free to two parts bound may perhaps be taken as a fair average of the values reported. Cholesterol is absorbed from the intestine<sup>37</sup> when fed in solution in oil or in natural solution as in egg yolk, and causes an increase in the cholesterol of the blood.<sup>38, 39</sup> Similar increases in the cholesterol of the blood after feeding fat alone have been reported<sup>26, 40, 41</sup> but these increases have not been confirmed in recent work.<sup>27</sup> Cholesterol has been reported to be increased in narcosis<sup>13, 42</sup> and in alcoholism,<sup>43</sup> also in pregnancy.<sup>23</sup> In pathological conditions cholesterol is increased in apparently all cases of jaundice,<sup>44</sup> presumably because of stoppage of one of its normal paths of excretion. In diabetes there is generally an increase, although as in the case of fat and lecithin the increases are not constant.<sup>45</sup> Increases have also been reported in nephritis<sup>22, 45-47</sup> and lues.<sup>34</sup> Decreases have been noted in cachexia of various origins.<sup>29, 46, 48</sup>

All of these lipoids are probably synthesized by the animal organism. The formation of fat from carbohydrate and probably also from protein is too well known to need discussion here. That "lecithin" is synthesized by the animal organism has been shown by McCollum<sup>49</sup> with rats, by Fingering<sup>50</sup> and McCollum<sup>51</sup> in feeding experiments with laying ducks and hens, and by

Bloor<sup>27</sup> in fat absorption experiments. As regards cholesterol, Gardner and Lander<sup>52</sup> have found that cholesterol is synthesized in the body of growing chicks, but not readily. Dezani<sup>53</sup> has claimed that it is readily synthesized in the animal body.

The functions of "lecithin" and cholesterol in the organism are little understood, although there can be no question regarding their importance since they are constituents of all living cells and probably constitute most of the "built in" or invisible fat of the tissues. Recent investigations<sup>27</sup> have shown that lecithin probably takes an active part in fat metabolism as the first stage through which the fats pass in their utilization by the organism.

As regards the function of cholesterol there is some evidence that it may serve as food. Mendel and Leavenworth<sup>54</sup> have shown that the cholesterol content of the developing hen's egg diminishes during development. Moreover, there appears to be a considerable cholesterol metabolism in the animal organism, since it or its products are always present in the feces in considerable quantities although it is quite readily absorbed from the intestine.<sup>37</sup> Cholesterol probably has also some function in fat metabolism because of the presence in the blood plasma and in most cells of cholesterol esters whose relation to the cholesterol—in the blood plasma, at least—is claimed to be constant;<sup>33</sup> also because in pathological conditions it increases or decreases along with the other lipoids, preserving a fairly constant relationship with them as has been indicated by the work of Fischer,<sup>17</sup> Müller,<sup>22</sup> Klemperer and Umber,<sup>21</sup> and as shown by the work below. Cholesterol is believed by Lifschütz<sup>55</sup> to be the source of the bile acids.

The presence of a cholesterase in blood corpuscles has been reported by Cytronberg.<sup>56</sup>

In all the work on the blood lipoids noted above there have been very few attempts to make complete analyses of these constituents and still fewer to correlate the results in an effort to determine the distribution of the lipoids between plasma and corpuscles or to discover relationships such as would be expected to occur among substances concerned with a common phase of metabolism. One effort in this direction is the work of Mayer and Schaeffer<sup>57</sup> in the elaboration of their theory of cellular constants. They found that in the red blood cells there is a constant

relationship between the fatty acids and the lipoid phosphorus on the one hand and between the cholesterol and the fatty acids on the other. A similar constancy of relationship between fatty acids and cholesterol in the blood has been reported by Terroine<sup>58</sup> who found besides that the relation remained constant during fat absorption.<sup>41</sup> A fairly constant relationship between total fatty acids and "lecithin" during fat absorption was noted by Bloor.<sup>27</sup>

Having, then, available suitable methods for the approximately complete analysis of the lipoid constituents of the blood, it seemed worth while to undertake in human beings a study of the distribution of the lipoids in both normal and pathological conditions in the effort to discover the normal values and relationships and how these are affected by various diseases, the whole to serve as a basis for future more detailed study of such conditions as show marked abnormalities.

*The blood of normal men examined was that of students in this school; that of normal women, of students in the nurses' training course of Simmons College. The pathological blood samples were obtained mainly from patients in the medical wards of the Peter Bent Brigham Hospital. For the work of obtaining most of these samples I am indebted to Dr. D. J. MacPherson of the Peter Bent Brigham Hospital, for those of normal men to Dr. O. F. Rogers formerly of this school. To all of these persons and to the authorities of the hospital I acknowledge my indebtedness and express my thanks for their help in making this work possible.*

Having in mind possible changes produced in the blood on standing, the samples were worked up as soon as possible, generally within 2 hours after being drawn from the vein. In the few cases where that was not possible the blood was kept on ice until used. All samples were obtained in the post-absorptive condition, before breakfast. Analyses both of whole blood and plasma were made and from these and the percentage of corpuscles, the lipoid values of the corpuscles were calculated. From the values so obtained the relations between the constituents in whole blood, plasma, and corpuscles were determined.

The routine procedure in the examination of the blood was much the same as that reported in earlier papers<sup>59</sup> and therefore need not be given in detail here. About 10 cc. of blood were drawn from one of the veins of the arm, and run into a gradu-



ated centrifuge tube containing two drops of saturated sodium citrate solution. After thoroughly mixing, a 3 cc. sample was measured out for "whole blood" and run into alcohol-ether as usual. The remainder was centrifuged at 3,800 R.P.M. for 10 minutes, the volume of corpuscles noted, and 3 cc. of the plasma were measured out into alcohol-ether as before.

For the determination of "total fat"<sup>59</sup> 10 cc. of the alcohol-ether extract were adequate in most instances but for the blood of anemia patients 15 cc. were necessary. The method used for the determination of cholesterol has recently been described.<sup>60</sup> 10 cc. of the extract were required for its determination in normal blood and quantities varying from 3 cc. (in severe diabetes) to 15 cc. (in anemia) in pathological blood. The method used for the determination of "lecithin" has also been described recently.<sup>61, 27</sup> In the present work the strychnine molybdate precipitation<sup>62</sup> was used throughout. 15 cc. of the blood extract were required for normal blood and from 10 (severe diabetes) to 25 cc. (anemia) in the pathological samples. The results of the analyses are given in the tables.

TABLE I.  
*Lipoids of Normal Blood, Gm. per 100 Cc.*

Corpuscles.	per cent	Total fatty acids.			Lecithin.			Cholesterol.			Fat.		Total fatty acids Lecithin			Lecithin Cholesterol			Total ether- soluble.	Plasma.
		Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.				
<i>Men.</i>																				
C. W. B. *		0.29	0.30	0.28	0.29	0.21	0.40	0.21	0.21	0.21	0.05	0.00	1.00	1.43	0.70	1.38	1.00	1.90	0.57	
D. E. C.		0.34	0.34	0.34	0.30	0.24	0.38	0.19	0.19	0.19	0.08	0.07	1.13	1.41	0.89	1.58	1.26	2.00	0.60	
R. D. C.		0.41	0.43	0.40	0.30	0.26	0.35	0.25	0.31	0.18	0.10	0.15	1.36	1.66	1.11	2.00	0.84	1.94	0.82	
E. M. D.		0.34	0.37	0.30	0.31	0.22	0.42	0.20	0.20	0.20	0.12	0.00	1.10	1.68	0.71	1.55	1.10	2.10	0.64	
M. F. S.		0.43	0.41	0.45	0.33	0.24	0.44	0.24	0.28	0.19	0.10	0.14	1.30	1.71	1.02	1.37	0.86	2.31	0.76	
J. R. L.		0.41	0.40	0.42	0.31	0.21	0.44	0.20	0.21	0.19	0.14	0.11	3.21	1.90	0.95	1.55	1.00	2.32	0.67	
R. MacL.		0.37	0.37	0.37	0.29	0.20	0.41	0.23	0.26	0.19	0.10	0.08	1.27	1.85	0.90	1.26	0.80	2.16	0.69	
M. M.		0.38	0.37	0.39	0.31	0.20	0.44	0.22	0.21	0.23	0.12	0.08	1.23	1.85	0.90	1.41	0.95	1.91	0.64	
C. D. M.		0.37	0.42	0.31	0.31	0.25	0.40	0.21	0.23	0.19	0.13	0.03	1.20	1.68	0.77	1.81	0.92	1.10	0.72	
E. P. S.		0.30	0.31	0.30	0.31	0.24	0.40	0.19	0.20	0.18	0.04	0.02	0.97	1.30	0.75	1.63	1.20	2.20	0.58	
F. S. T.		0.41	0.42	0.40	0.29	0.22	0.38	0.20	0.21	0.19	0.16	0.13	1.41	1.90	1.05	1.45	1.05	2.00	0.70	
H. J. W.		0.33	0.35	0.31	0.30	0.19	0.42	0.19	0.21	0.18	0.12	0.02	1.10	1.84	0.70	1.57	0.90	2.30	0.62	
B. P. H.		—	0.37	—	0.29	0.22	0.38	0.20	0.21	0.19	0.11	—	—	1.70	—	1.45	1.05	2.00	0.65	
H. M. E.		—	0.41	—	—	0.23	—	—	0.21	—	0.14	—	—	1.80	—	—	1.10	—	0.69	
<i>Women.</i>																				
Miss R.	38	0.39	0.44	0.30	0.31	0.22	0.46	0.24	0.26	0.22	0.16	0.00	1.26	2.00	0.65	1.30	0.85	2.10	0.77	
" T.	39	0.34	0.39	0.27	0.28	0.21	0.39	0.22	0.22	0.22	0.13	0.00	1.21	1.86	0.69	1.28	0.95	1.80	0.67	
" K.	40	0.35	0.41	0.27	0.29	0.19	0.43	0.23	0.25	0.20	0.15	0.00	1.20	2.10	0.63	1.26	0.76	2.15	0.71	
" D.	39	0.37	0.39	0.34	0.29	0.19	0.44	0.22	0.25	0.18	0.12	0.03	1.27	2.00	0.77	1.32	0.76	2.44	0.69	
" F.	38	0.40	0.45	0.32	0.29	0.18	0.48	0.24	0.24	0.24	0.20	0.00	1.40	2.50	0.67	1.21	0.75	2.00	0.74	
" Alb.	35	0.38	0.40	0.32	0.28	0.17	0.48	0.21	0.22	0.19	0.17	0.00	1.36	2.35	0.67	1.33	0.77	2.52	0.67	

TABLE 1—Concluded.

	Corpuscles <sup>10</sup>	Total fatty acids.			* Lecithin			Cholesterol.			Fat.		Total fatty acids Lecithin			Lecithin Cholesterol			Total other- soluble.
		Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	
	per cent																		
Mrs. M.....	37	0.42	0.47	0.33	0.29	0.21	0.43	0.22	0.24	0.20	0.20	0.03	1.45	2.23	0.77	1.32	0.87	2.15	0.77
Miss Rl.**.....	37	0.33	0.35	0.30	0.26	0.15	0.44	0.16	0.15	0.17	0.18	0.00	1.27	2.33	0.08	1.02	1.00	2.60	0.54
" A.**.....	39	0.35	0.38	0.30	0.25	0.14	0.42	0.16	0.15	0.18	0.21	0.00	1.40	2.70	0.71	1.56	0.93	2.33	0.57
Averages,																			
Men.....		0.36	0.38	0.36	0.30	0.22	0.40	0.21	0.22	0.19	0.11	0.07	1.20	1.08	0.89	1.44	0.96	2.08	0.67
Variations { High.....		0.41	0.43	0.45	0.33	0.26	0.44	0.25	0.31	0.23	0.16	0.15	1.41	1.00	1.14	1.63	1.26	2.32	0.82
Low.....		0.29	0.30	0.28	0.20	0.20	0.35	0.19	0.19	0.17	0.01	0.00	0.97	1.41	0.70	1.20	0.84	1.90	0.57
Women.....		0.36	0.40	0.29	0.29	0.19	0.44	0.23	0.21	0.21	0.16	0.01	1.31	2.15	0.69	1.29	0.82	2.14	0.72
Variations { High.....		0.42	0.47	0.31	0.31	0.22	0.48	0.24	0.26	0.24	0.20	0.03	1.45	2.70	0.77	1.33	0.95	2.60	0.77
Low.....		0.32	0.35	0.27	0.28	0.17	0.39	0.21	0.21	0.19	0.12	0.00	1.20	1.80	0.63	1.21	0.75	1.80	0.57

The value "total fatty acids" is obtained by subtracting the value for cholesterol from that of "total fat" as determined. The value "lecithin" is obtained by multiplying the value obtained for phosphoric acid (lipoid) by 8. "Fat" in the plasma is obtained by subtracting the fatty acids combined as "lecithin" ( $0.70 \times$  the lecithin value) together with those combined as cholesterol esters ( $0.48 \times$  the cholesterol value) from the "total fatty acids" and multiplying the result by 1.05; in the corpuscles, by subtracting the fatty acids combined as "lecithin" from "total fatty acids" and multiplying by 1.05 as before. These calculations are made on the assumptions (1) that the lecithin is oleo-stearyl-lecithin; (2) that two-thirds of the cholesterol of the plasma is combined with fatty acid (oleic acid); and (3) that in the corpuscles all the cholesterol is free—assumptions which are believed to be sufficiently near the truth for the purposes of this work. The values given for corpuscles are calculated from those of the whole blood and plasma, taking into account the percentage of corpuscles. The value "total ether-soluble" (plasma) is obtained by adding together the values for "lecithin," cholesterol (including esters), and "fat." The values of the various constituents in plasma and whole blood are believed to be within 5 per cent of the true value; in the corpuscles, since they are calculated values, the variations are greater. Ratios between total fatty acids and lecithin,  $\frac{\text{total fatty acids}}{\text{lecithin}}$ , and between lecithin and cholesterol,

$\frac{\text{lecithin}}{\text{cholesterol}}$ , are included in the table, the first because of the relationship observed between "total fatty acids" and "lecithin" during fat absorption,<sup>27</sup> leading to the belief that lecithin is a stage in fat metabolism through which the fats must pass, and the second because of the antagonistic behavior of these two substances observed in several biological processes.<sup>63-65</sup>

\* In the case of the normal men where the corpuscle percentage was not determined, 43 per cent (the value given by Keith, Rowntree, and Geraghty<sup>66</sup> as the average for normal men) was used in the calculations. This average value for normal student blood has since been found correct in determinations on other students' blood.

\*\* The lipoids of these two samples were determined last and therefore after this blood had stood for a longer time than the others. Since the values for all the lipoids are lower in these samples it seemed probable that these low values may have been due to enzyme action and they are therefore not included in the averages.

TABLE II.

## Lipoids of Pathological Blood, Gm. per 100 Cc.

	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Fat.			Total fatty acids Lecithin*			Lecithin Cholesterol		Total ether- soluble.
		Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	Corpus- cles.	Whole.	Plasma.	
<i>Women.</i>	<i>per cent</i>																		
1. Mitral stenosis.....	44	0.50	0.46	0.55	0.30	0.13	0.45	0.23	0.27	0.18	0.20	0.23	1.67	2.55	1.22	1.30	0.67	2.50	0.78
17. Diabetes.....	38	0.35	0.35	0.35	0.25	0.15	0.44	0.16	0.16	0.16	0.17	0.04	1.40	2.30	0.80	1.50	0.90	2.70	0.56
47. ".....	41	0.34	0.36	0.31	0.32	0.17	0.53	0.25	0.27	0.22	0.21	0.09	1.06	2.10	0.60	0.66	1.00	2.10	0.69
2. ".....	46	0.52	0.57	0.46	0.37	0.30	0.46	0.30	0.38	0.21	0.18	0.13	1.40	1.90	1.00	1.20	0.79	2.20	1.05
70. ".....	47	0.56	0.70	0.40	0.49	0.48	0.50	0.54	0.65	0.41	0.04	0.05	1.14	1.50	0.87	1.22	0.74	1.20	1.50
3. Debility.....	44	0.47	0.48	0.46	0.32	0.17	0.51	0.19	0.19	0.19	0.26	0.10	1.47	2.82	0.90	1.70	0.90	2.70	0.71
4. Carcinoma, anemia.....	23	0.51	0.58	0.28	0.22	0.23	0.19	0.28	0.25	0.38	0.30	0.15	2.32	2.52	1.47	0.79	0.92	0.50	0.90
12. ".....	36	0.30	0.33	0.27	0.30	0.24	0.40	0.24	0.19	0.33	0.07	0.00	1.38	0.68	1.25	1.26	1.21		0.59
6. ".....	34	0.40	0.44	0.32	0.20	0.17	0.26	0.21	0.22	0.19	0.21	0.14	2.00	2.60	1.23	0.95	0.78	1.37	0.71
7. Myocarditis.....	45	0.41	0.35	0.46	0.29	0.21	0.37	0.26	0.25	0.27	0.08	0.21	1.67	1.24	1.11	0.84	1.37		0.66
8. Malnutrition.....	33	0.39	0.26	0.65	0.28	0.20	0.44	0.22	0.25	0.16	0.00	0.34	1.40	1.30	1.48	1.27	0.80	2.80	0.57
9. Nephritis.....	37	0.53	0.53	0.53	0.33	0.22	0.52	0.24	0.25	0.22	0.26	0.17	1.60	2.41	0.98	1.38	0.88	2.36	0.85
10. Syphilis, alcoholic.....	34	0.38	0.36	0.42	0.22	0.19	0.28	0.18	0.14	0.26	0.15	0.22	1.73	1.90	1.50	1.22	1.36	1.61	0.55
11. Goiter.....	43	0.40	0.45	0.33	0.28	0.23	0.35	0.19	0.20	0.18	0.15	0.09	1.42	2.00	0.94	1.47	1.15	1.94	0.68
5. Asthma.....	33	0.52	0.46	0.64	0.26	0.14	0.49	0.20	0.17	0.26	0.28	0.34	2.00	3.30	1.31	1.30	0.82	1.90	0.67
15. Anemia.....	12	0.43	0.44	0.36	0.14	0.12	0.29	0.12	0.11	0.19	0.30	0.15	3.00	3.67	1.20	1.20	1.10	1.50	0.59
16. Pernicious anemia.....	17	0.41	0.44	0.26	0.21	0.18	0.36	0.19	0.18	0.23	0.22	0.00	1.95	2.43	0.72	1.10	1.00	1.13	0.67
21. ".....	25	0.44	0.41	0.53	0.24	0.15	0.51	0.21	0.21	0.21	0.16	0.17	1.83	2.73	1.04	1.14	0.70	2.43	0.62
82. ".....	10	0.37	0.37	0.37	0.16	0.14	0.34	0.16	0.16	0.16	0.19	0.13	2.31	2.64	1.10	1.00	0.88	2.12	0.57
20. Splanchnoptosis.....	50	0.34	0.43	0.25	0.27	0.20	0.34	0.22	0.25	0.19	0.17	0.01	1.26	2.15	0.74	1.23	0.80	1.80	0.74
<i>Men.</i>																			
13. Carcinoma, anemia.....	28	0.36	0.41	0.23	0.24	0.20	0.34	0.20	0.21	0.16	0.17	0.00	1.50	2.05	0.70	1.20	0.98	2.12	0.63
14. Pernicious anemia.....	11	0.42	0.42	0.42	0.15	0.12	0.39	0.15	0.15	0.15	0.25	0.14	2.80	3.50	1.08	1.00	0.80	2.60	0.60
103. Hemophilia.....	34	0.39	0.35	0.47	0.27	0.14	0.50	0.17	0.17	0.17	0.17	0.12	1.41	2.41	0.94	1.00	0.85	2.94	0.56
109. ".....	38	0.37	0.37	0.27	0.16	0.15	0.19	0.16	0.16	0.16	0.17	0.06	1.37	2.31	0.82	1.42	0.84	2.47	0.61
167. Adulterated albumen.....	48	0.46	0.52	0.40	0.31	0.20	0.43	0.21	0.23	0.19	0.26	0.10	1.80	2.00	0.93	1.49	0.83	2.39	0.82

## DISCUSSION.

*Lipoids of Normal Blood.*—For reasons discussed in the earlier part of the paper a detailed comparison of the normal values recorded above with those in the literature is not considered profitable. The "total fatty acids" and "total ether-soluble" values fall within the wide limits given in the literature. The "lecithin" is higher than the values given in the earlier literature but is about the same as that more recently reported.<sup>67</sup> The cholesterol values are higher than most of the reported values (which average about 0.17 gm. per 100 cc.),<sup>34</sup> a fact that is partly at least accounted for by the method used which, as has been noted before,<sup>60</sup> gives higher values than the methods in common use.

The variations from the average of the various constituents are from 10 to 20 per cent, being greatest for "total fatty acids" and least for "lecithin."

The differences in the content of lipoids of men's and women's blood is not great, and it is possible that a more extended series of analyses may eliminate these differences. The total fatty acids of the women's plasma is slightly higher than that of the men, while that of the corpuscles is somewhat lower. The same is true of the glycerides. Cholesterol and "total ether-soluble" are practically the same in both. "Lecithin" is lower in the plasma and higher in the corpuscles of the women than of the men.

The "lecithin" content of the corpuscles is always greater than that of the plasma, approximately double, while the cholesterol and total fatty acid content of the corpuscles is about the same, although generally a little less.

The value for "lecithin" in the corpuscles is generally about twice that of cholesterol,  $\frac{L.}{C.} = 2.08$ , average, in men and 2.14 in women, while in the plasma the values for the two constituents are nearly equal,  $\frac{L.}{C.} = 0.96$  for men and 0.82 for women. The constancy of these ratios, which holds also for most of the pathological blood (see below), suggests a definite relationship between these substances.

In the blood both of men and women the normal cholesterol value is about one-third that of the "total ether-soluble." The lecithin is about the same fraction of the "total ether-soluble" in men's blood but is a little lower, about one-fourth of this value in women's blood.

The value for "fat" (which represents all the fatty acid not combined as lecithin) in the corpuscles is generally zero in women's blood and frequently so in men's, making it probable that all the cholesterol of the corpuscles is free (not combined with fatty acid). The absence of fat from the corpuscles raises the question whether there is any present in the normal plasma. Letsche<sup>6</sup> found none. The insolubility of fat in water and the fact that such a small amount of suspended fat is needed to make water cloudy while normal plasma is almost always clear would seem to give some support to this finding. On the assumption made above that two-thirds of the cholesterol of the plasma is combined, the amounts of glycerides in the plasma are small. If we assume further, as Klemperer<sup>7, 21</sup> has done, that all the cholesterol in the plasma is combined the glycerides will be reduced to amounts which are probably not far from the limit of error of the determinations. This is a point of considerable importance in determining the significance of lipemia (milky of the plasma) in pathological conditions, also in determining how the fat is normally transported in the blood, especially in fasting, when considerable quantities of fat are passing and yet the plasma remains clear. It seems unlikely, on the other hand, that there is no free cholesterol in the plasma, for if that were the case it would be difficult to explain the action of cholesterol as an antihemolytic against several agents<sup>68</sup> which appear to react only with free cholesterol.

### *Lipoids of Pathological Blood.*

#### *A. Summary of Important Data Regarding the Individual Samples.*

1. *Mitral Stenosis.*—Total fatty acids increased, correspondingly increased values for "fat," "total ether-soluble," and  $\frac{\text{T.F.A.}}{\text{L.}}$

17, 47, 2, 70. *Diabetes.*—Nos. 17 and 47 practically normal (cholesterol low in 17). In Nos. 2 and 70, all the constituents

were much increased, although the increases in total fatty acids are relatively less than in lecithin and cholesterol. The plasma in Case 47 was somewhat cloudy; in all the others it was clear.

3. *Debility*.—"Total fatty acids," "fat," and the ratio  $\frac{T.F.A.}{L.}$  slightly high, especially in the corpuscles. Lecithin and  $\frac{L.}{C.}$  in corpuscles slightly increased.

4. *Carcinoma of Stomach, Anemia*.—"Total ether-soluble" high, "total fatty acids" and cholesterol high in corpuscles, "lecithin" low in corpuscles, "fat" high in both. Marked disturbance of the ratios  $\frac{T.F.A.}{L.}$  and  $\frac{L.}{C.}$  in the corpuscles.

12. *Carcinoma of Peritoneum, Transverse Colon, Uterus, and Stomach*.—Blood lipoids practically normal. Increase in cholesterol in corpuscles.

6. *Carcinoma of Stomach, Hernia of Abdominal Wall, Hematuria*.—Lipoids practically normal. Lecithin somewhat low, especially in corpuscles.

7. *Chronic Myocarditis, Hypertension*.—No marked disturbance in the distribution of the lipoids. "Total fatty acids" relatively lower in plasma and higher in corpuscles than normal.

8. *Malnutrition*.—"Total fatty acids" and "fat" low in plasma and high in corpuscles. "Total ether-soluble" low.

9. *Chronic Nephritis, Chronic Myocarditis, Hypertension*.—"Total ether-soluble" high; "total fatty acids" and "fat" high in both plasma and corpuscles. "Lecithin" increased also, but to a less extent. The ratios, however, fall within the limits of normal variation. Plasma cloudy.

10. *Syphilis, Chronic Alcoholism*.—"Total fatty acids," "fat," and cholesterol high in corpuscles; "lecithin" and cholesterol low in plasma. "Total ether-soluble" low.  $\frac{T.F.A.}{L.}$  in corpuscles double the normal value. Plasma cloudy.

11. *Goiter, Hyperthyroidism (?)*.—Lipoids normal.

5. *Bronchial Asthma (?)*.—"Total fatty acids" and "fat" high, resulting in a high  $\frac{T.F.A.}{L.}$  ratio.



15. *Anemia Due to Bothriocephalus Latus*.—"Lecithin" low throughout; cholesterol very low in plasma. "Fat" high in both plasma and corpuscles. The ratio  $\frac{T.F.A.}{L.}$  is very high throughout;  $\frac{L.}{C.}$  in the corpuscles is low.

16. *Pernicious Anemia*.—Cholesterol low in plasma. Lecithin low in corpuscles. Plasma slightly cloudy.

21. *Pernicious Anemia, Hemorrhagic Stomatitis*.—Cholesterol and "lecithin" low in plasma. "Total fatty acids" high in corpuscles, and relatively high in plasma.  $\frac{T.F.A.}{L.}$  high in both corpuscles and plasma.

82. *Pernicious Anemia*.—Lecithin and cholesterol low throughout. Plasma cloudy.

20. *Splanchnoptosis*.—Lipoids practically normal. Plasma milky. (This blood was not obtained until it was 24 hours old.)

13. *Anemia (?)*, *Carcinoma of Stomach (?)*.—"Lecithin" low throughout.

14. *Syphilis, Pernicious Anemia*.—"Total fatty acids" slightly high. "Lecithin" very low in plasma, cholesterol low in plasma and corpuscles. "Fat" high in plasma.  $\frac{T.F.A.}{L.}$  high throughout.

103 and 109. *Hemophilia*.—"Lecithin" low in plasma. Cholesterol also low, but little if any beyond the bounds of normal variation.

87. *Addison's Disease*.—Lipoids practically normal. Fat slightly high in plasma. Total ether-soluble high but within the normal limits.

Of these samples the following are practically normal as regards both the amounts and distribution of the blood lipoids: Diabetes (mild) (17 and 47); carcinoma of peritoneum (12); carcinoma of stomach (6); chronic myocarditis (7); goiter (11); splanchnoptosis (20); Addison's disease (87).

### B. Summary of Data Regarding the Individual Lipoids.

#### "Total Fatty Acids."

*Increased*.—Mitral stenosis (1) (corpuscles); diabetes (2 and 70); debility; chronic myocarditis (7) (corpuscles); malnutrition (8)

(corpuscles); chronic nephritis (9); alcoholism (10); bronchial asthma (5); carcinoma of stomach (4); pernicious anemia (21), pernicious anemia (14), Addison's disease (87) (plasma).

*Decreased.*—Chronic myocarditis (plasma).

### "Lecithin."

*Increased.*—Diabetes (2 and 70) (plasma); chronic nephritis (9) (corpuscles).

*Decreased.*—Diabetes (17) (plasma); carcinoma, anemia (4) (corpuscles); carcinoma, hemophilia (6); anemia (15); pernicious anemia (16) (corpuscles); pernicious anemia (21) (plasma); pernicious anemia (82); carcinoma, anemia (13) (plasma); syphilis, pernicious anemia (14) (plasma); hemophilia (103 and 109) (plasma).

### Cholesterol.

*Increased.*—Diabetes (2) (plasma), (70) (plasma and corpuscles); carcinoma, anemia (4) (corpuscles); carcinoma of peritoneum (12).

*Decreased.*—Diabetes (17) (plasma and corpuscles); malnutrition (8) (corpuscles). Syphilis, alcoholic (10) (plasma); bronchial asthma (5) (plasma); anemia (15) (plasma); pernicious anemia (82) (plasma and corpuscles); syphilis, anemia (14) (plasma and corpuscles); hemophilia (103) (plasma).

### "Fat."

*High.*—Mitral stenosis (1) (corpuscles); diabetes (47) (plasma); debility (3) (plasma); carcinoma, anemia (4) (plasma and corpuscles); carcinoma, hemophilia (6) (plasma and corpuscles); chronic nephritis (9) (plasma and corpuscles); syphilis, alcoholic (10) (corpuscles); bronchial asthma (5) (plasma and corpuscles); anemia (15) (plasma and corpuscles); anemia (16) (plasma); anemia (21) (corpuscles); anemia (82) (corpuscles); Addison's disease (87) (plasma).

*Low.*—Carcinoma of peritoneum (12) (plasma); malnutrition (8) (plasma). "Total ether-soluble" is outside the normal limits of variation in only five cases. It is high in diabetes (2 and 70), carcinoma (4), and chronic nephritis (9); low in alcoholism (10).

*C. Discussion of Lipoids of Pathological Blood.*

The constituent most often abnormal in value is the "fat" which is increased in fifteen and decreased in two of the twenty-five samples examined. The next in frequency of variation is the "lecithin," decreased in thirteen and increased in three. Then come the "total fatty acids," increased in twelve and decreased in one; then cholesterol which is increased in four cases and decreased in nine; and finally the "total ether-soluble" which is outside the normal limits of variation in only five samples.

Abnormal values for cholesterol and "lecithin" occur most frequently in the plasma—the composition of the corpuscles tending to remain constant with regard to these constituents. Variations of "total fatty acids" and "fat" occur with almost equal frequency in plasma and corpuscles. The increases in "total fatty acids" in general result from increases in "fat," the notable exceptions being the diabetics (2 and 70) where the "lecithin" and presumably also the cholesterol esters contribute. The increase of "total fatty acids" in the corpuscles is relative as well as absolute, the ratio  $\frac{T.F.A.}{L.}$  being high in fifteen out of the twenty-five samples. The low lecithin values in many cases also increase the value of the ratio. In the plasma the ratio  $\frac{T.F.A.}{L.}$  is abnormal in eleven samples.

The ratio  $\frac{L.}{C.}$  shows frequent abnormality in the corpuscles but is remarkably constant in the plasma, being outside the normal figures in only six cases out of the twenty-five. The constancy of the ratio suggests strongly a close relationship between the two. "Lecithin" has been found to increase during fat absorption by all who have investigated this point,<sup>27</sup> and "lecithin" therefore is definitely indicated as a step in fat metabolism. Cholesterol has been reported to increase similarly, and although this finding has not been corroborated by the author's recent work (at least during the first 8 hours after feeding), the constancy of the relationship  $\frac{L.}{C.}$  which persists in pathological conditions suggests strongly that cholesterol (as its esters ?) also has a part in fat metabolism, although presumably at a later stage than "lecithin."

The above results indicate that the most characteristic features of pathological conditions are the increase in the "fat" of plasma and corpuscles and decrease of "lecithin" of the plasma. These are particularly interesting in view of the above finding that lecithin is probably a stage in the metabolism of the fats and that the lecithin formation takes place in the corpuscles, and would seem to indicate a diminished activity of this mechanism in diseased conditions. Where the increase in fat is not accompanied by abnormalities in the lecithin values, it might be interpreted as the result of increased mobilization.

Low cholesterol values occur almost always in those conditions where the vitality is low, as in blood samples 8, 10, 15, 82, 14, a finding which is in agreement with those reported in the literature.<sup>29, 46, 48</sup> In this connection the recent work of McCrudden<sup>71</sup> is interesting. In a case of muscular weakness which he studied he found a relation between the severity of the condition and the low values for cholesterol and blood sugar. As the patient improved under treatment, the values for both blood sugar and cholesterol increased. Whether there is any connection between the cholesterol and blood sugar could of course only be settled by extended study, but the fact that the cholesterol is high in severe diabetes (70) where the blood sugar is also high (0.26 per cent) is suggestive.

With such limited data only the most general inferences can be made regarding individual conditions. In mild diabetes (17 and 47, urine free from sugar and diacetic acid) the values for the blood lipoids are normal or below normal throughout and with no notable variations from the normal ratios. In severe diabetes (2 and 70) in relatively good clinical condition (urine containing small amounts of sugar but no diacetic acid) all the blood lipoids are increased—up to 100 per cent or more, but the ratios are again not notably disturbed. In none of these cases was there any lipemia (in the sense of cloudy or milky appearance of the plasma) in spite of the high lipid values. The constancy of the ratios with the lack of visible fat in the plasma would seem to indicate that in diabetes under good treatment the fat-utilizing mechanism is functioning in a practically normal manner, although requiring in the severe cases a high lipid "pressure" to keep it going. The increase of "lecithin" and cholesterol along with the

fat is in agreement with the findings of Fischer<sup>17</sup> and Klemperer<sup>7, 21</sup> in diabetes.

In anemia the low values for cholesterol in the plasma are not without significance, in view of the part which cholesterol is said to play in protecting the corpuscles from the action of hemolytic agents, as suggesting a possible reason for the destruction of the corpuscles in these conditions.

In hemophilia the low values for "lecithin" are suggestive in view of the connection of cephalin with blood clotting shown by Howell<sup>60</sup> and the more recent observations of Hurwitz and Lucas<sup>72</sup> that cephalin injected into the circulation increases the speed of clotting, also that local applications of cephalin to wounds quickly stops bleeding.

#### SUMMARY.

The term "lipoids" is used in this paper as a general term for all those substances connected with the metabolism of the fatty acids, including the fatty acids, their naturally occurring compounds, and such substances as cholesterol which occur naturally in combination with the fatty acids and which are therefore presumably connected with their metabolism. Reasons for the choice of the term are given.

Complete analyses of the lipoids of the blood of twenty-three normal and twenty-five abnormal persons are presented, together with ratios between certain of these lipoids. The more important results are as follows:

*Normal Blood.*—The "lecithin" content of the corpuscles is found to be approximately double that of the plasma, while the cholesterol and "total fatty acid" values are almost always lower in the corpuscles than in the plasma. The value for "lecithin" in the corpuscles is generally about twice that of cholesterol, while in the plasma their values are nearly equal. The ratio between these constituents ( $\frac{L}{C}$ ) is quite constant in normal blood (especially plasma) and remains so in most of the pathological samples, suggesting a definite relationship between these constituents, and making it probable that cholesterol (as its esters?) has a part in fat metabolism.

In the blood both of men and women the normal cholesterol value is about one-third that of the "total ether-soluble." The

"lecithin" value is about the same fraction in men but is a little lower (one-fourth) in women. The amount of fat (glycerides) present in normal plasma is small. In the corpuscles it may be entirely absent.

*Pathological Blood.*—The blood lipoids are normal throughout in only eight of the twenty-five samples, while the "total ether-soluble" (representing what is ordinarily termed "fat") is within normal limits in twenty out of the twenty-five. The importance of the complete lipid analysis in investigating abnormal conditions as against the ordinary determination of "fat" is thus emphasized.

The most characteristic feature of pathological conditions in human beings is the increase of "total fatty acids" and "fat" both in plasma and corpuscles, and the decrease of "lecithin" in the plasma. Since the "fat" is probably to be regarded as the inactive form of the body lipoids, the form in which they are stored—the raw material of fat metabolism—and the "lecithin" as the first step in its utilization, an undue accumulation of "fat" or a notably decreased value for "lecithin" probably indicates a diminished activity of the fat metabolism. The ratio ( $\frac{L}{C}$ ) in the plasma is constant and normal in all but six of the pathological samples.

In severe diabetes the blood lipoids are all greatly increased but the ratios between these constituents are practically normal. In mild diabetes the blood lipoids are practically normal.

The low values for the cholesterol of the plasma in anemia are suggestive in view of the antihemolytic function ascribed to cholesterol.

The low values for "lecithin" (which includes cephalin) in the plasma of hemophilia are noteworthy in view of the connection recently shown between cephalin (thromboplastic substance of Howell) and blood coagulation.

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# THE EFFECT ON NITROGEN PARTITION OF SUBSTITUTING ALCOHOL FOR SUCROSE IN AN OTHERWISE FIXED DIET.

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## INTRODUCTION.

In view of the great number of published reports on the physiological effect of alcohol, any further effort may seem superfluous. As far as I have been able to determine, however, there has never been published a report of the effect on nitrogen partition of substituting for sucrose an isodynamic amount of alcohol during an otherwise fixed diet. That alcohol under certain conditions causes a diminution in the total nitrogen excretion, in others an increase, and in some cases no change at all, is well known, and has been fully discussed by Atwater.<sup>1</sup>

Salant and Hinkel<sup>2</sup> were the first to report detailed results of the effect of alcohol on the composition of urine. They used dogs exclusively as subjects and studied the problem from the standpoint of adding alcohol to the diet. They determined the amount and ratios present of total nitrogen, phosphates, chlorides, and the various forms of sulfur.

In the same year Mendel and Hilditch<sup>3</sup> published the results of their work on the effect on nitrogen partition of adding alcohol to an otherwise fixed diet. They used men and dogs as subjects. They determined the total nitrogen, urea, ammonia, uric acid, purine-base, creatine, and creatinine nitrogen.

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<sup>1</sup> Atwater, W. O., and others, *Physiological Aspects of the Liquor Problem*, Boston, 1903, ii, 169.

<sup>2</sup> Salant, W., and Hinkel, F. C., *J. Pharm. and Exp. Therap.*, 1909-10, i, 493.

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## 602 Alcohol Effect on Nitrogen Partition

These two series of investigations admirably complete the study of the effect of alcohol on the composition of the urine when the alcohol is added to the diet. However, they leave undetermined the effect on nitrogen partition of substituting isodynamic quantities of alcohol for sucrose during an otherwise fixed diet.

### EXPERIMENTAL PROCEDURE.

The subjects used in these experiments were two men, A and B. Both subjects were not unaccustomed to the mild use of alcoholic beverages. The diet was the same for both and consisted of the following foods in the amounts indicated. As far as possible the same grade of food was consumed by both subjects throughout.

Breakfast, 7 a. m.		Lunch, 12 m.		Dinner, 6 p. m.	
	gm.		gm.		gm.
Grapenuts.....	30	Bread.....	150	Potato.....	100
Orange juice.....	250	Butter.....	30	Steak.....	100
Bread.....	50	Ham.....	33	Corn (canned)....	100
Butter.....	15	Apple pie.....	100	Bread.....	50
Sugar.....	30	Sugar.....	30	Butter.....	30
Whole milk, cc....	325			Sugar.....	30

Water 2,000 cc. per day for A and 1,500 cc. per day for B.

Using Locke's<sup>4</sup> tables as a basis of calculation this diet yields about 2,800 calories and contains about 14 gm. of nitrogen.

At the beginning of the experiment A weighed 50 kg. At its termination he weighed 51 kg. With A, the experiment was divided into three periods, a preliminary period of 4 days and a terminal period of 3 days during which the diet was as given above, and an intermediate period of 4 days' duration. The dietary change consisted in substituting 40 cc. of rum for the 30 gm. of sucrose in each meal.

At the beginning of the experiment B weighed 70 kg. At its termination his weight was the same. With B the experiment

<sup>4</sup> Locke, E. A., Food Values, New York, 1915.

was likewise divided into three periods, consisting in this case of preliminary and terminal periods of 3 days each, during which the diet was as given above, and the intermediate period of 3 days' duration, the dietary change being the same as for A.

The total nitrogen, ammonia, uric acid, creatinine, and creatine were determined according to the methods of Folin and his collaborators. The urea was determined according to Marshall.<sup>5</sup>

## RESULTS.

The ingestion of the alcohol produced a pharmacological response consistent with the quantity and individual tolerance.

The analytical results are shown in Tables I and II. The quantities noted represent the amounts of the various nitrogenous constituents excreted per 24 hours.

TABLE I.  
*Composition of Urine of A.*

			Nitrogen partition						In terms of total N.				
Date	Vol- ume	Alcohol (45 per cent)	Total N	Urea N	NH <sub>3</sub> N	Creatinine N	Uric acid N	Undetermined N	Urea N	NH <sub>3</sub> N	Creatinine N.	Uric acid N.	Undetermined N.
1916	cc	cc	gm	gm	gm	gm	gm	gm	per cent	per cent	per cent	per cent	per cent
Mar. 14	1,730		8 98	6 87	0 32	0 41	0 14	1 24	76 5	3 6	4 6	1 6	13 7
" 15	1,720		9 29	6 98	0 34	0 44	0 14	1 39	75 1	3 7	4 7	1 5	15 0
" 16	2,000		9 66	7 57	0 34	0 43	0 12	1 20	78 3	3 5	4 4	1 2	12 6
" 17	2,050		9 43	7 10	0 33	0 44	0 13	1 43	75 2	3 5	4 5	1 4	15 4
" 18	1,760	120	9 69	7 12	0 29	0 41	0 13	1 74	73 5	3 0	4 2	1 3	18 0
" 19	2,000	120	9 17	7 21	0 30	0 45	0 14	1 07	78 6	3 3	4 9	1 5	11 7
" 20	1,860	120	9 30	7 21	0 31	0 42	0 14	1 23	77 5	3 3	4 6	1 5	13 2
" 21	1,820	120	9 18	7 24	0 29	0 43	0 12	1 10	78 8	3 2	4 6	1 3	12 1
" 22	1,720		9 05	7 02	0 29	0 42	0 13	1 19	77 6	3 2	4 7	1 4	13 1
" 23	1,820		9 10	7 64	0 29	0 41	0 12	0 64	83 9	3 2	4 5	1 3	7 1
" 24	1,850		9 22	7 26	0 29	0 42	0 14	1 11	77 7	3 2	4 6	1 6	12 9

<sup>5</sup> Marshall, E. K., Jr., *J. Biol Chem*, 1913, xv, 487.



## VICINE AND DIVICINE.

By P. A. LEVENE AND JAMES K. SENIOR.

PLATE 6.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 31, 1916.)

Recent developments in the chemistry of the nucleic acids have renewed the interest of chemists in vicine, discovered by Ritthausen<sup>1</sup> over 40 years ago. Schulze and Trier,<sup>2</sup> on the basis of Ritthausen's analytical data, were able to recognize in vicine a pyrimidine glucoside. As such, the substance resembled the pyrimidine nucleosides entering into the composition of nucleic acid. As a hexoside, it had a possible bearing on the nucleic acids of the animal type. The fact that plant nucleic acids containing hexoses have not yet been discovered, added still more mystery and interest to the subject; hence the simultaneous activity of several laboratories in solving the problem of the structure of vicine and divicine.

Chronologically, the first paper after that of Schulze and Trier was one by Treat B. Johnson.<sup>3</sup> Arguing from Ritthausen's divicine data and from personal experience with Traube's<sup>4</sup> 4,5-diamino-2,6-dioxypyrimidine, Johnson came to the conclusion that the two bases were identical. In the course of work on Traube's<sup>5</sup> diaminopyrimidines,

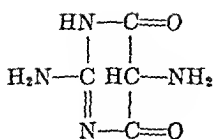
<sup>1</sup> Ritthausen, H., and Kreusler, U., *J. prakt. Chem.*, 1870, ii, 336. Ritthausen, H., *ibid.*, 1873, vii, 374; 1881, xxiv, 202; 1899, lix, 480. Ritthausen, H., and Preuss, *ibid.*, 487. Ritthausen, *Ber. chem. Ges.*, 1876, ix, 301; 1896, xxix, 894, 2108.

<sup>2</sup> Schulze, E., and Trier, G., *Z. physiol. Chem.*, 1910-11, lxx, 143.

<sup>3</sup> Johnson, T. B., *J. Am. Chem. Soc.*, 1914, xxxvi, 337.

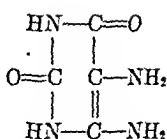
<sup>4</sup> Traube, W., *Ber. chem. Ges.*, 1900, xxxiii, 1382.

<sup>5</sup> Traube, *Ber. chem. Ges.*, 1893, xxvi, 2556.



2,5-Diamino-4,6-dioxypyrimidine.

and



4,5-Diamino-2,6-dioxypyrimidine.

Johnson and Johns<sup>6</sup> discovered two reactions. The first led to the formation of uric acid by fusion of the 4,5-diamino base with urea; the second enabled them to distinguish an amino group in the 5-position by its color reaction with molybdic acid solution. E. Fischer<sup>7</sup> used the first of the two Johnson reactions to test the structure of divicine, but found that, on fusion with urea, it behaved differently from the 4,5-diamino base. He also observed that the two bases differed in their water of crystallization. He was, however, so much impressed by the logic of Johnson's arguments that he accepted the 4,5-diamino-2,6-dioxypyrimidine structure for divicine, attributing the differences between the two substances to stereoisomerism. Thannhauser and Dorfmueller<sup>8</sup> also accepted Johnson's views and proceeded to prepare the nucleoside synthetically—they supposed, with success.

Preceding Fischer's publication by approximately 10 weeks, appeared a note on vicine by Levene.<sup>9</sup> He reported experimental facts which led him to conclude that divicine was the 2,5- not the 4,5-diamino base. His conclusions were drawn from the similarity between divicine and the 2,5-diamino base in respect to water of crystallization and behavior towards urea and towards nitrous acid. The 4,5-diamino base differs in all three points from both divicine and the 2,5-diamino base. In a private letter, Fischer accepted the conclusions of Levene.

However, the entire question of the structure of vicine and divicine did not seem definitely solved. The views of Levene as to the positions of the amino groups were based on analogy in the behavior of the molecules as wholes. Direct chemical evidence

<sup>6</sup> Johnson, T. B., and Johns, C. O., *J. Am. Chem. Soc.*, 1914, xxvi, 545, 970.

<sup>7</sup> Fischer, E., *Ber. chem. Ges.*, 1914, xlvii, 2611.

<sup>8</sup> Thannhauser, S. J., and Dorfmueller, G., *Ber. chem. Ges.*, 1915, xlvii, 1304.

<sup>9</sup> Levene, P. A., *J. Biol. Chem.*, 1914, xviii, 305.

was lacking. The present investigation furnishes such evidence regarding the location of the two amino groups.

The presence of one amino group in the 2-position was demonstrated by the oxidation of divicine to guanidine. Many years ago Fischer<sup>10</sup> obtained guanidine on oxidizing 2-imidouric acid with potassium chlorate. It was expected that 2,5-diamino-4,6-dioxypyrimidine would yield guanidine under the same conditions. The expectation was realized, and thus direct proof was furnished of the presence of an amino group in the 2-position.

Until now, the color test of Johnson has been the only evidence for the location of the second amino group. This evidence was also substantiated by direct chemical proof. Both divicine and the synthetic 2,5-diamino base were converted into 2-imidopseudouric acid, which possessed a very definite crystalline form, and physical properties differing from those of the 4-imidopseudouric acid obtained from the 4,5-diamino base.

It was intended to convert both imidopseudouric acids into purine derivatives. 2-Imidouric acid was expected from one, uric acid from the other. The conversion into uric acid of 4-imidopseudouric acid was accomplished quite readily. Indeed this substance is probably an intermediate product in the uric acid synthesis of Johnson. The conversion into uric acid of 2-imidopseudouric acid, obtained from either divicine or the synthetic 2,5-diamino base, proved impractical under the conditions of the present work. The reactions, however, gave no uric acid.

Occasion was taken to repeat the condensation of 4,5-diamino-2,6-dioxypyrimidine with urea. In Levene's previous work, the substances prepared under the conditions described by Johnson and Johns, although quite granular and non-hygroscopic, were amorphous. Johnson and Johns made no reference to the crystalline character of their substance. Since a small amount of amorphous material had also been obtained by condensing urea with the 2,5-diamino base, it was concluded to make an effort to crystallize the substances formed by the condensation of each of the three bases with urea. Pure crystalline uric acid was obtained from the 4,5-diamino base, after the reaction product had been repeatedly dissolved in alkali and reprecipitated by the

<sup>10</sup> Fischer, *Ber. chem. Ges.*, 1897, xxx, 571.



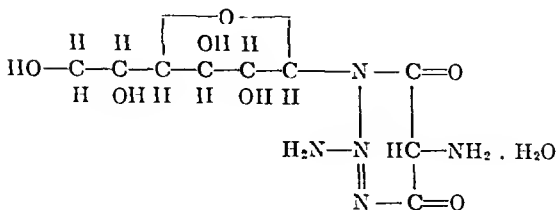
addition of acid. From the other two bases, it was very difficult or impossible to prepare a crystalline condensation product, and when it was obtained its crystalline form differed entirely from that of either uric acid or 2-imidopseudouric acid.

The composition of the sulfates was also redetermined, since the analytical data previously reported were unsatisfactory. In spite of many recrystallizations, it was not possible to get perfectly definite results. Apparently the sulfates underwent hydrolysis in the course of purification. Fischer, too, had arrived at the same conclusion regarding the sulfate of divicine. However, in a general way, the impression was substantiated that the sulfate of the 4,5-diamino base crystallizes with more water of crystallization than does that of the 2,5-diamino base, and that in this respect the sulfate of divicine more closely resembles the latter.

Hence, the structure of the base may be regarded as conclusively established. Divicine is 2,5-diamino-4,6-dioxypyrimidine.

Passing from the base to the whole vicine molecule, it appeared that even the most recent publications had left the data very incomplete. There remained discrepancies between the analytical values published by Ritthausen and the values required by the formulas suggested by either Johnson or Levene. Furthermore, Ritthausen had great difficulty in preparing different samples of vicine of constant composition. Perhaps on this account Fischer considered the possibility of its being a polynucleoside. An effort was therefore made to determine the conditions necessary to prepare perfectly pure vicine. These were attained when the substance was recrystallized from water and dried without driving off its single molecule of water of crystallization. Ritthausen, who was the only worker besides the authors to publish analyses of vicine, recrystallized his material from alcoholic solutions. Partial dehydration probably caused his irregular results.

In addition, the monomolecular nature of the nucleoside was demonstrated by molecular weight determinations. This evidence also shows that only one hexose and one pyrimidine are present in the molecule of vicine. The hexose was shown independently by Levene and by Fischer to be *d*-glucose. The following formula for vicine is therefore suggested.



Vicine.

There remain two points to be cleared up in regard to the structure of vicine; the particular atom of the pyrimidine base to which the sugar is joined in glucosidic union remains unknown, and the  $\alpha$  or  $\beta$  nature of this glucosidic linkage is still to be determined. Work on these questions is now in progress.

#### EXPERIMENTAL PART.

*Preparation of Vicine.*—Only two slight changes were made in the method of preparing vicine described by Levene.<sup>9</sup> 3 per cent sulfuric acid was used instead of 5 per cent in the extraction of the vetch meal. The filtrate from the mercuric sulfide was concentrated to small volume, filtered, and cooled. The vicine crystallized out from the aqueous solution. The mother liquors were worked up for a further yield by the method of alcoholic extraction. In this way, a yield of about 2.5 gm. of vicine per kg. of vetch seeds was obtained. The substance was purified by recrystallizing it from water. Bone-black was used as a decolorizer, and the amount of water necessary was 20 cc. per gm. of vicine. Two such treatments gave a product that was almost snow-white and pure enough for all but analytical purposes.<sup>11</sup>

*Analysis of Vicine.*—Vicine contains one molecule of water of crystallization. At 100° *in vacuo* over sulfuric acid, it loses this water very slowly, at 135° the loss is much more rapid, and at 155° there is a slight discoloration before equilibrium is attained. Assuming the mononucleosidic formula, and adding to it one

<sup>11</sup> To obtain the lipoids from vetch seeds, Dr. C. J. West extracted the seeds several times with 95 per cent alcohol. This extract was fractionated, and one fraction found to consist largely of vicine. When purified by recrystallization from water, it proved to be identical with the material prepared as above.

molecule of water, the calculated analysis of vicine agrees very closely with the figures found. A sample of the substance prepared by the method described above was recrystallized four times from water, and dried by exposure to the air. When further dried at room temperature *in vacuo* over sulfuric acid, its weight remained constant.

0.1032 gm. substance gave 0.1401 gm.  $\text{CO}_2$  and 0.0523 gm.  $\text{H}_2\text{O}$ .

0.1012 " " required 12.45 cc. of 0.1 N HCl.

0.0991 " " " 12.15 " " 0.1 " "

A portion of this material, when heated to  $61^\circ$  *in vacuo* over  $\text{H}_2\text{SO}_4$ , remained constant in weight. Another sample, when heated to  $100^\circ$  under the same conditions, lost weight very slowly. Two portions were then heated to constant weight *in vacuo* over sulfuric acid, and the percentage of water lost was determined.

I This portion came to constant weight after 12 hours at  $135^\circ$ . There was no discoloration of the material. 0.5174 gm. substance lost 0.0255 gm weight

II This portion came to constant weight after 18 hours at  $155^\circ$ . The material was very slightly discolored. 1.0177 gm. substance lost 0.0555 gm weight

	Calculated for $\text{C}_8\text{H}_{12}\text{N}_4\text{O}_5 \cdot \text{H}_2\text{O}$ :	Found.
C	37.24	37.02
H	5.63	5.67
N	17.39	17.24 17.15
$\text{H}_2\text{O}$	5.59	I. 5.51 II. 5.78

I 0.1015 gm. dry substance gave 0.1442 gm.  $\text{CO}_2$  and 0.0491 gm.  $\text{H}_2\text{O}$

0.1023 " " " required 13.35 cc. 0.1 N HCl.

0.1014 " " " " 13.10 " 0.1 " "

II 0.1043 " " " gave 0.1489 gm.  $\text{CO}_2$  and 0.0595 gm  $\text{H}_2\text{O}$

0.1049 " " " required 13.50 cc. 0.1 N HCl.

0.1008 " " " " 13.15 " 0.1 " "

	Calculated for $\text{C}_8\text{H}_{12}\text{N}_4\text{O}_5$ :	I.	Found:	II
C	39.45	38.75		38.94
H	5.30	5.41		5.42
N	18.42	18.29 18.10	18.19	18.28

*Molecular Weight of Vicine.*—The molecular weight of vicine agrees with the mononucleosidic formula. The fact that the substance is monomolecular shows that there can be only one sugar

and only one pyrimidine group present in the unhydrolyzed compound. The sample used for the determinations was purified by repeated crystallization from water, and dried at room temperature *in vacuo* over sulfuric acid. The Beckmann boiling point method was used, with water as the solvent.

0.843 gm. substance in 19.46 gm.  $H_2O$  gave a rise of  $0.070^\circ$ .

0.498 " " " 19.49 " " " " " "  $0.044^\circ$ .

Assuming, on the basis of the analyses, that the substance contained 5.6 per cent water of crystallization,

0.796 gm. dehydrated substance in 19.51 gm.  $H_2O$  gave a rise of  $0.070^\circ$ .

0.470 " " " 19.52 " " " " " "  $0.044^\circ$ .

	Calculated for $C_{16}H_{18}N_4O_7$	Found:
Mol. Wt.....	304	302 284

*Preparation of the Sulfates of the Three Bases.*—These substances were found to have anomalous compositions. In order to recrystallize them, it was necessary to dissolve them in hot dilute solutions of sulfuric acid, and to precipitate them by adding alcohol to the cold, filtered solutions. The samples were air-dried. Even so, when this method was repeated several times in what seemed to be an exactly similar fashion, it was impossible to obtain sulfate of 2,5-diamino-4,6-dioxypyrimidine of constant composition. The analyses given are typical of the material used in the experiments later described.

*4,5-Diamino-2,6-dioxypyrimidine Sulfate.*—This substance was prepared by the method of Traube.<sup>4</sup> After several recrystallizations in the manner described above, a substance of constant composition was obtained.

0.1974 gm. substance gave 0.1671 gm.  $CO_2$  and 0.0775 gm.  $H_2O$ .

0.1008 " " required 19.25 cc. 0.1 N HCl.

0.1000 " " 19.15 " 0.1 " "

0.2024 " " gave 0.1048 gm.  $BaSO_4$ .

0.2007 " " 0.1043 " "

	Calculated for $(C_6H_6N_4O_2)_2 \cdot H_2SO_4 \cdot 2H_2O$	Calculated for $(C_6H_6N_4O_2)_2 \cdot H_2SO_4 \cdot 4H_2O$	Found:
C.....	22.95	21.13	23 09
H.....	4.34	4.88	4 39
N.....	26.80	24 65	26 75 26 83
$H_2SO_4$ .....	23.45	21 59	21 76 21 84

*2,5-Diamino-4,6-dioxypyrimidine Sulfate*.—This substance was prepared by the method of Traube,<sup>5</sup> and recrystallized several times in the manner described above.

- a. 0.1056 gm. substance required 21.10 cc. 0.1 N HCl.  
 b. 0.0990 " " " 19.80 " 0.1 " "

The substance was recrystallized under conditions apparently exactly similar to the previous ones.

- c. 0.1009 gm. substance required 20.75 cc. 0.1 N HCl.  
 d. 0.0980 " " " 20.20 " 0.1 " "

	Calculated for (C <sub>4</sub> H <sub>6</sub> N <sub>4</sub> O <sub>2</sub> ) <sub>2</sub> H <sub>2</sub> SO <sub>4</sub> ·0.5H <sub>2</sub> O:	Calculated for (C <sub>4</sub> H <sub>6</sub> N <sub>4</sub> O <sub>2</sub> ) <sub>2</sub> H <sub>2</sub> SO <sub>4</sub> ·H <sub>2</sub> O:	Found:	
N.....	28.65	28.00	a. 27.99	b. 28.02
			c. 28.83	d. 28.83

*Divicine Sulfate*.—5 gm. of vicine were placed with 10 cc. of 20 per cent sulfuric acid in an unstoppered Erlenmeyer flask which was then immersed in boiling water. The vicine dissolved within a minute or two. After 8 minutes' heating a crystalline precipitate began to form, and at the end of 15 minutes the contents of the flask were a thick paste. The flask was removed, its contents were diluted with alcohol, and the vessel was cooled in ice. The precipitate was filtered out and dried. The yield was about 3 gm. No attempt was made to obtain this substance of constant composition. After recrystallization in the manner described above, a sample analyzed as follows:

- 0.1024 gm. substance gave 0.0934 gm. CO<sub>2</sub> and 0.0350 gm. H<sub>2</sub>O.  
 0.1000 " " required 19.25 cc. 0.1 N HCl.  
 0.0999 " " " 19.30 " 0.1 " "  
 0.3014 " " gave 0.1809 gm. BaSO<sub>4</sub>.  
 0.3009 " " " 0.1799 " "

	Calculated for (C <sub>4</sub> H <sub>6</sub> N <sub>4</sub> O <sub>2</sub> ) <sub>2</sub> H <sub>2</sub> SO <sub>4</sub> ·0.5H <sub>2</sub> O:	Calculated for (C <sub>4</sub> H <sub>6</sub> N <sub>4</sub> O <sub>2</sub> ) <sub>2</sub> H <sub>2</sub> SO <sub>4</sub> ·2H <sub>2</sub> O:	Found:	
C.....	24.53	22.95	24.89	
H.....	3.86	4.34	3.82	
N.....	28.65	26.80	26.97	27.07
H <sub>2</sub> SO <sub>4</sub> .....	25.07	23.45	25.22	25.12

*Oxidation of the Sulfates*.—The oxidation of the sulfates was carried out by a method analogous to the one used by Fischer<sup>10</sup> in the oxidation of 2-imidouric acid. 2.4 gm. of the sulfate

were placed in a bottle with 18 cc. of hydrochloric acid solution made up of equal volumes of acid of sp. gr. 1.19 and of water. The bottle was shaken for 2 hours. During the first hour and a half, 0.5 gm. of potassium chlorate was added to the mixture. In no case did complete solution occur. The reaction mixtures were filtered and cooled. With the sulfate of the 4,5-diamino base, no precipitation occurred. With the sulfates of the 2,5-diamino base and divicine, crystalline precipitates formed. These were filtered out. All three solutions were then evaporated to dryness *in vacuo*. The solid residues were taken up with alcohol, and the insoluble materials filtered out. Then the alcoholic filtrates were again evaporated to dryness *in vacuo*. The solid residues were dissolved in a few cc. of water, made alkaline with a little sodium hydroxide, and the solutions tested for the presence of guanidine.

*KClO<sub>3</sub> and 4,5-Diamino-2,6-dioxypyrimidine Sulfate.*—Sodium picrate was added to the solution obtained from the sulfate of 4,5-diamino-2,6-dioxypyrimidine by the method just described. There was no precipitate. Guanidine is therefore not formed in the reaction.

*KClO<sub>3</sub> and 2,5-Diamino-4,6-dioxypyrimidine and Divicine Sulfates.*—Sodium picrate was added to the solutions obtained from the sulfates of 2,5-diamino-4,6-dioxypyrimidine and divicine by the method just described. In each case a yellow precipitate appeared. These precipitates were filtered out and washed on the filter with water and ether. They were then dissolved in boiling water, and a little bone-black was added. The solutions were filtered and cooled. The precipitates which formed had the crystalline form found by Emich<sup>12</sup> to be characteristic of guanidine picrate. Both precipitates were recrystallized from alcohol and dried *in vacuo* over sulfuric acid at 106°.

a. Substance from 2,5-diamino-4,6-dioxypyrimidine sulfate.

0.0864 gm. substance gave 22.2 cc. of N at 26.1° and 759 mm.

b. Substance from divicine sulfate.

0.0868 gm. substance gave 22.05 cc. N at 26.4° and 766.5 mm.

	Calculated for C <sub>7</sub> H <sub>8</sub> N <sub>6</sub> O <sub>7</sub> ·	Found:
N.....	29.18	a. 29.28    b. 29.21

<sup>12</sup> Emich, F., *Monatsh. Chem.*, 1892, xii, 23.

These figures are considered to prove that the substance is in both cases guanidine picrate.

The formation of guanidine picrate from divicine and 2,5-diamino-4,6-dioxypyrimidine, and its non-formation from 2,6-dioxy-4,5-diaminopyrimidine show that divicine has an amino group in the 2-position.

*Treatment of the Sulfates with Urea.*—This work is a repetition and confirmation of the work of Johnson and Johns<sup>6</sup> and Fischer.<sup>7</sup> In each case, 1 gm. of the sulfate was triturated with 1.5 gm. of urea and heated in a hard glass test-tube for 1 hour at 160–170°. The melt was then cooled and dissolved out with water. In every case the reaction mixture contained some insoluble material.

*Urea and 4,5-Diamino-2,6-dioxypyrimidine Sulfate.*—To the reaction mixture obtained from 2 gm. of material by the method described above, potassium hydroxide was added until the solid was nearly all dissolved. The solution was then acidified with acetic acid. A heavy amorphous precipitate appeared. This was filtered out, dissolved in 200 cc. of hot dilute potassium hydroxide, and poured into 250 cc. of hot 6 per cent hydrochloric acid. The precipitate formed was perfectly crystalline. It was filtered out, washed with water and alcohol, and dried *in vacuo* over sulfuric acid. The yield was 1.1 gm. The substance gave the murexide test with nitric acid, and formed with silver nitrate a white complex insoluble in ammonia.

0.1613 gm. substance gave 0.2115 gm. CO<sub>2</sub> and 0.0380 gm. H<sub>2</sub>O.  
0.1003 “ “ required 23.85 cc. 0.1 N HCl.

	Calculated for C <sub>4</sub> H <sub>4</sub> N <sub>2</sub> O <sub>2</sub>	Found:
C.....	35.69	35.76
H.....	2.40	2.64
N.....	33.34	33.31

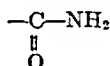
These results show that the substance is uric acid.

*Urea and Divicine and 2,5-Diamino-4,6-dioxypyrimidine Sulfates.*—In both cases a little acid was added to the turbid reaction mixtures obtained by the method described above, with the effect of increasing the amounts of precipitate. These were filtered out, boiled with bone-black in a little water, and the solutions filtered and cooled. The precipitates formed were filtered out and each dissolved in a little boiling water. In each case, the

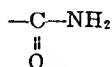
filtered solution gave a very slight precipitate on cooling. In the case of the substance from 2,5-diamino-4,6-dioxypyrimidine sulfate, this precipitate, although crystalline, did not in the least resemble the characteristic needles of 2-imidopseudouric acid. In the case of the substance from divicine sulfate the final precipitate was completely amorphous. No trace of uric acid was observed in either case.

The formation of uric acid by fusion with urea strikingly differentiates the sulfate of the 4,5-diamino base from the sulfates of divicine and the 2,5-diamino base.

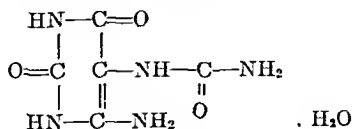
*Preparation of Imidopseudouric Acids from the Sulfates.*—Both of the synthetic sulfates, on being treated with potassium cyanate, yielded an imidopseudouric acid crystallizing with one molecule of water. One of them, the 2-imidopseudouric acid made from the 2,5-diamino base, has already been described by Traube;<sup>13</sup> the other, the 4-imidopseudouric acid from the 4,5-diamino base, is reported here for the first time. Analysis showed that the new compound contained a molecule of water of crystallization and was isomeric with the one described by Traube. Its conversion into uric acid (to be described later) showed that the



group must be attached to the amino group in either the 4- or the 5-position. Since the compound, like Traube's imidopseudouric acid, gave no blue color with molybdic acid in alkaline solution (Johnson's test for an amino group in the 5-position), it was concluded that the



group was attached to the amino group in the 5-position, and the following structural formula was assigned to the compound:



<sup>13</sup> Traube, *Ber. chem. Ges.*, 1893, xxvi. 2558.



The differences noted between the two substances were as follows:

*2-Imidopseudouric Acid.*

1. Crystallized in long needles (Fig. 1).
2. At 100°, 1 liter of water dissolved 1.5 gm.
3. Lost its water of crystallization in 8 hours at 135° *in vacuo* over sulfuric acid.

*4-Imidopseudouric Acid.*

- Crystallized in oblong plates (Fig. 2).
- At 100°, 1 liter of water dissolved 7 gm.
- Lost its water of crystallization in 40 hours at 135° *in vacuo* over sulfuric acid.

In all three respects, the substance obtained from the sulfate of divicine (Fig. 3) was identical with the substance obtained from the sulphate of the 2,5-diamino base. This evidence confirms the view that divicine has an amino group in the 5-position. At the same time, certain differences in behavior between the sulfate of divicine and that of the 2,5-diamino base were noted.

*Sulfate of the 2,5-Diamino Base.*

1. When this substance was poured into boiling KOCN solution, a marked purple color appeared, which only vanished after being boiled some 10 minutes.
2. The yield of 2-imidopseudouric acid obtained from the above reaction was about 0.55 gm. per gm. of sulfate used.

*Sulfate of Divicine.*

- When this substance was poured into boiling KOCN solution, only faint and fugitive traces of purple color could be observed.
- The yield of 2-imidopseudouric acid obtained from the above reaction was about 0.04 gm. per gm. of sulfate used.

Concerning the cause of these differences no explanation can be offered. The method of preparing the imidopseudouric acid was the same in all three cases. 2 gm. of sulfate were poured into a boiling solution of 10 gm. of potassium cyanate in 20 cc. of water. After about 2 minutes' boiling, 4 gm. of solid potassium cyanate were added and the boiling was continued for 10 minutes more, or, in the case of the sulfate of the synthetic 2,5-diamino base, until the purple color had vanished. Then the hot reaction mixture was poured into an excess of hot 5 per cent hydrochloric acid.

1. *4-Imidopseudouric Acid from the Sulfate of the 4,5-Diamino Base.*—When the hot acid solution, obtained as described above.

was cooled, a crystalline precipitate, only slightly contaminated by amorphous material, appeared at once. This was filtered out and dried. The yield was about 0.45 gm. per gm. of sulfate used. The substance was then boiled with bone-black in water, using about 135 cc. of water per gm. of material. When the filtered solution was cooled, the substance crystallized out almost colorless. This product was recrystallized four times from water, the hot solution being filtered each time.

2. *2-Imidopseudouric Acid from the Sulfate of the 2,5-Diamino Base.*—When the hot acid solution, obtained as described above, was cooled, a crystalline precipitate, only slightly contaminated by amorphous material, appeared at once. This was filtered out and dried. The yield was about 0.55 gm. per gm. of sulfate used. The substance was then boiled with bone-black in water, using about 650 cc. of water per gm. of material. When the filtered solution was cooled, the substance crystallized out snow-white. This product was recrystallized twice from water, the hot solution being filtered each time.

3. *2-Imidopseudouric Acid from the Sulfate of Divicine.*—When the hot acid solution, obtained as described above, was cooled, the crystalline precipitate only appeared after several days' standing, and was then contaminated by much amorphous impurity. The substance was filtered out and dissolved in boiling water, using about 650 cc. per gm. of material. The precipitate formed on cooling was filtered out, and the process repeated once or twice more until the pure white material showed no trace of amorphous impurity. The substance was then filtered out and dried. The yield was about 0.04 gm. per gm. of sulfate used. This product was recrystallized four times from water, the hot solution being filtered each time.

In all three cases, the final materials were dried *in vacuo* over sulfuric acid at room temperature. No one of them gave a blue color with molybdic acid in ammoniacal solution. Portions of all three substances were heated at 135° *in vacuo* over sulfuric acid. The 4-imidopseudouric acid came to constant weight after 40 hours of heating; the two samples of 2-imidopseudouric acid after 8 hours.

4-Imidopseudouric acid from the sulfate of the 4,5-diamino base.

0.1024 gm. substance gave 0.1112 gm.  $\text{CO}_2$  and 0.0408 gm.  $\text{H}_2\text{O}$ .

a. 0.0750 " " required 18.60 cc. 0.1 N HCl.

b. 0.0755 " " " 18.65 " 0.1 " "

0.3969 " " lost 0.0341 gm. weight when heated.

Dehydrated material.

a. 0.0736 gm. substance required 19.75 cc. 0.1 N HCl.

b. 0.0772 " " " 20.65 " 0.1 " "

2-Imidopseudouric acid from the sulfate of the 2,5-diamino base.

0.1038 gm substance gave 0.1126 gm.  $\text{CO}_2$  and 0.0416 gm.  $\text{H}_2\text{O}$ .

a. 0.0749 " " required 18.30 cc. 0.1 N HCl.

b. 0.0755 " " " 18.55 " 0.1 " "

0.5191 " " lost 0.0466 gm. weight when heated.

Dehydrated material.

a. 0.0791 gm. substance required 21.20 cc. 0.1 N HCl.

b. 0.0930 " " " 26.40 " 0.1 " "

2-Imidopseudouric acid from the sulfate of divicine.

0.1008 gm substance gave 0.1082 gm.  $\text{CO}_2$  and 0.0413 gm.  $\text{H}_2\text{O}$ .

a. 0.0523 " " required 12.85 cc. 0.1 N HCl.

b. 0.0503 " " " 12.30 " 0.1 " "

0.1004 " " lost 0.0088 gm. weight when heated.

Dehydrated material.

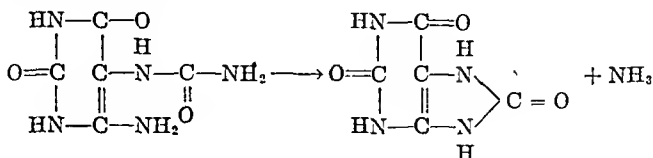
a. 0.0455 gm substance required 12.25 cc. 0.1 N HCl.

b. 0.0461 " " " 12.35 " 0.1 " "

	Calculated for $\text{C}_5\text{H}_7\text{N}_5\text{O}_2 \cdot \text{H}_2\text{O}$ :	Found:			
		Substance from sulfate of 4, 5 diamino base	Substance from sulfate of 2, 5 diamino base	Substance from sulfate of divicine	
C	29.54	29.62	29.59	29.39	
H	4.47	4.45	4.48	4.60	
N	34.49	a.34.74 b.34.60	a.34.23 b.34.42	a.34.42	b.34.26
$\text{H}_2\text{O}$	8.87	8.59	8.98	8.8	
	For $\text{C}_5\text{H}_7\text{N}_5\text{O}_2$				
N...	37.84	a.37.59 b.37.48	a.37.55 b.37.74	a.37.71	b.37.53

*Conversion of the Pseudouric Acids into Uric Acids.*—It was originally intended to convert the 4-imidopseudouric acid and the 2-imidopseudouric acid from both the synthetic and the natural pyrimidines into uric acid and 2-imidouric acid, respectively. But the very poor yields of 2-imidopseudouric acid obtained from the sulfate of divicine prevented the carrying out of the latter half of this plan.

*Uric Acid from 4-Imidopseudouric Acid.*—This conversion was carried out by a method similar to that used by Fischer<sup>10</sup> in converting 2-imidopseudouric acid into 2-imidouric acid, the only difference being that a molecule of ammonia instead of a molecule of water was eliminated.



1 gm. of 4-imidopseudouric acid was sealed in a bomb tube with 3 cc. of concentrated hydrochloric acid, and heated for 2 hours at 120°. The tube was constantly shaken during this period. When cooled and opened, its contents consisted of a brownish granular mass insoluble in water. This substance was dissolved in dilute sodium hydroxide, the solution boiled with bone-black, filtered, and poured into an excess of hot, dilute hydrochloric acid. There was an immediate crystalline precipitate. This was cooled, filtered out, and dried. The yield was 0.6 gm. The substance was boiled for 20 minutes with 100 cc. of 5 per cent hydrochloric acid, and filtered out. Then the purification process was repeated once more in an exactly similar fashion. The final material was perfectly crystalline and almost pure white. It was filtered out, washed with water, and dried *in vacuo* over sulfuric acid at 106°. The substance gave the murexide test with nitric acid, and formed with silver nitrate a white complex insoluble in ammonia.

0.0995 gm. substance gave 0.1305 gm. CO<sub>2</sub> and 0.0233 gm. H<sub>2</sub>O.

0.0754 " " required 18.00 cc. 0.1 N HCl.

0.0766 " " " 18.25 " 0.1 " "

	Calculated for C <sub>5</sub> H <sub>4</sub> N <sub>4</sub> O <sub>3</sub> :	Found:
C.....	35.69	35.77
H.....	2.40	2.62
N.....	33.34	33.45 33.38

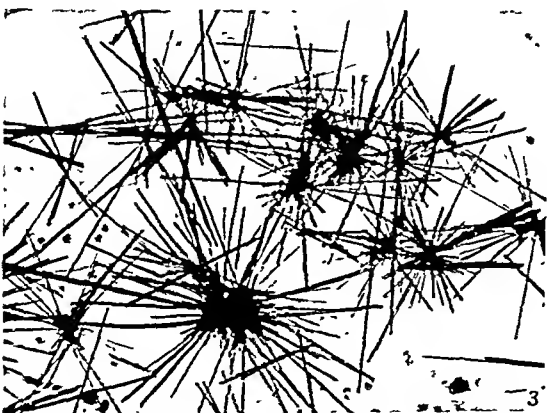
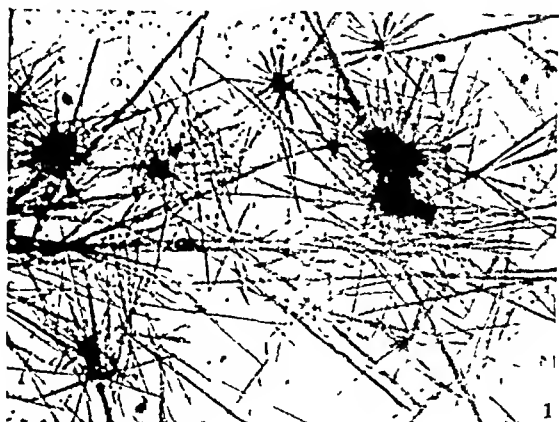
These data are considered to prove that the substance is uric acid.

## EXPLANATION OF PLATE 6.

FIG 1 2-Imidopseudouric acid from the sulfate of 2,5-diamino-4,6-dioxypyrimidine

FIG 2 4-Imidopseudouric acid from the sulfate of 4,5-diamino-2,6-dioxypyrimidine

FIG 3 2-Imidopseudouric acid from the sulfate of divicine.



(Levene and Senior : Vicine and Divicine )



# THE PREPARATION OF GUANIDINE SULFATE.

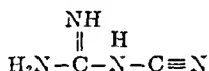
By P. A. LEVENE AND JAMES K SENIOR.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication May 31, 1916 )

In the course of work on divicine<sup>1</sup> it was necessary to prepare large quantities of a guanidine salt from which free guanidine could easily be obtained. The sulfate and carbonate suggested themselves, since both would give the free base on being treated with barium hydroxide. As no convenient method for preparing these salts could be found in the literature, it was necessary to devise one. This was successfully accomplished, and the preparation is here described in the hope that it may be of use to others.

The starting material was dicyandiamide



which was easily prepared from crude commercial calcium cyanamide by the method of Soll and Stutzer.<sup>2</sup> The yield was about 155 gm. per kg. of crude material.

0.0523 gm. substance required 24.75 cc. 0.1 N HCl.  
0.0515 " " " 24.30 " 0.1 " "

	Calculated for C <sub>2</sub> H <sub>4</sub> N <sub>4</sub>	Found
N.....	66.65	66.30 66.11

100 gm. of dicyandiamide were mixed in a 2 liter wide-mouthed round-bottomed flask with 200 cc. of 75 per cent sulfuric acid. After about 2 minutes the substance grew very hot, and there was a violent evolution of carbon dioxide which lasted for several

<sup>1</sup> Levene, P. A., and Senior, J. K., *J. Biol. Chem.*, 1916, **xxv**, 607.

<sup>2</sup> Soll, J., and Stutzer, A., *Ber. chem. Ges.*, 1910, **xlii**, 4533.



minutes. When this spontaneous reaction had nearly ceased, the flask was gently heated on a Babo funnel for 5 minutes, at a temperature sufficient to keep a good stream of gas coming off. The flask was then allowed to cool. At the moment when the contents began to solidify, 1,500 cc. of 95 per cent alcohol were added. The mixture was then cooled in the ice chest over night. The solid material (which consisted of almost pure ammonium sulfate) was filtered out and washed with 95 per cent alcohol. The filtrate and washings were combined and concentrated to a volume of about 300 cc. The liquid was then transferred to a large flask on the steam bath, and, while constantly stirred, was neutralized with barium carbonate. The solid barium salts were then filtered out. The liquid, which was usually a trifle alkaline, was just neutralized with dilute sulfuric acid, and concentrated to a volume of about 200 cc. It was then filtered and further concentrated to a thick oil. This oil was poured into 1 liter of 95 per cent alcohol. When stirred and scratched, it slowly solidified into a crystalline mass. The flask was cooled in the ice chest, the solid filtered out, washed with 95 per cent alcohol, and dried in the air bath at 100°. The yield was 125 gm.

- a. 0.0501 gm substance required 13.00 cc. 0.1 N HCl.  
 b. 0.0509 " " " 13.15 " 0.1 " "  
 a. 0.3008 " " gave 0.3162 gm. BaSO<sub>4</sub>.  
 b. 0.3023 " " " 0.3175 " "

	Calculated for (CN <sub>2</sub> H <sub>3</sub> ) <sub>2</sub> H <sub>2</sub> SO <sub>4</sub> :	Found	
		a	b
N	38.88	36.35	36.20
H <sub>2</sub> SO <sub>4</sub>	45.36	44.17	44.13

The nature of the impurities present was never determined as the substance was sufficiently pure for the desired purposes, and was used without further refinement.

# THE PITUITARY GLAND. ITS EFFECT ON GROWTH AND FISSION OF PLANARIAN WORMS.

By ROSALIND WULZEN.

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(Received for publication, May 2, 1916.)

The recorded effects of the pituitary gland on growth and reproduction are so varied that further investigation of the subject seems to be desirable.

Many<sup>1</sup> have found an inhibition in growth. On the other hand, Robertson and Burnett<sup>2</sup> report acceleration in growth of earcinomata, and Robertson<sup>3</sup> gives convincing experiments pointing to an early retardation and a later acceleration of growth in white rats. Goetsch<sup>4</sup> also reports increase in growth with rats under pituitary feeding. Some have observed in addition a sexual effect. Cushing<sup>5</sup> points out the wide occurrence of sexual disturbances as an accompaniment of pituitary disorders in clinical cases. He and also Aschner<sup>6</sup> find that the operative removal of the pituitary in young animals leads to sexual infantilism. Behrenroth<sup>7</sup> and Goetsch<sup>4</sup> have observed precocious sexual development accompanying treatment with the pituitary gland. Clark<sup>7</sup> gets increased egg production of hens on feeding pituitary. Pearl,<sup>1</sup> on the other hand, finds that in pullets pituitary substance does not bring about an earlier activation of the ovary than occurs in controls.

A local planarian worm *Planaria maculata* was selected as a good subject for experimentation because it will live and thrive

<sup>1</sup> Cerletti, U., *Arch. ital. biol.*, 1907, xlvii, 123. Sandri, O., *ibid.*, 1909, li, 337. Goetsch, E., and Cushing, H., cited by Cushing, *The Pituitary Body and Its Disorders*, Philadelphia, 1912, 11. Aldrich, T. B., *Am. J. Physiol.*, 1912, xxx, 352; 1912-13, xxxi, 94. Wulzen, R., *ibid.*, 1914, xxxiv, 127. Pearl, R., *J. Biol. Chem.*, 1916, xxiv, 123.

<sup>2</sup> Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280.

<sup>3</sup> Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 385.

<sup>4</sup> Goetsch, E., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 29.

<sup>5</sup> Aschner, B., *Arch. ges. Physiol.*, 1912, cxlvi, 1.

<sup>6</sup> Behrenroth, E., *Deutsch. Arch. klin. Med.*, 1914, cxiii, 393.

<sup>7</sup> Clark, L. N., *J. Biol. Chem.*, 1915, xxii, 485.

upon a meat diet exclusively, and because its small size makes possible the administration of a constant excess of food. Also the readiness with which it reproduces in the laboratory makes easy the study of the effects of diet upon this function. Child,<sup>8</sup> who has made exhaustive studies of the process of fission in *Planaria*, holds that the formation of a new worm from the piece resulting from fission "is as truly a reproduction as the process of the formation of a planarian from the egg." Hence changes in the rate of fission would indicate changes in the reproductive function of the worms just as surely as would changes in the rate of production of offspring by a mammal.

After being collected the worms were fasted in the laboratory for at least 2 weeks. In the second series very small worms were used. These were obtained by allowing tips cut from the posterior ends of fasting worms to regenerate. They then had a length of 3 mm. or less, and according to Child<sup>8</sup> must be considered, as very young worms. The animals were kept in finger bowls and the water upon them was often changed. They were fed almost without exception four times a week and were always given more than they could consume. The food was left before them over night. Throughout the experiment they gathered upon all varieties of meat given them and ate greedily. Data were collected upon the occurrence of fission, changes in length, and the final weight. The worms were measured in a flat-bottomed dish under which was a rule that could be moved. They were stirred to stimulate them to move with the maximum extension, and as they passed over the lines of the ruler they were measured. While this method is not exact because of the varying tonus of the worms, the measurements obtained indicate the size with an accuracy amply sufficient for the purposes of the experiment.

#### EXPERIMENTAL.

In the first series the worms were divided into three lots: those below 5 mm., those between 5 and 10 mm., and the largest ones, which measured a little below or above 10 mm. Each lot was again divided into two equal portions, one of which was fed upon pars glandularis of ox pituitary and the other upon liver. The

<sup>8</sup> Child, C. M., *Arch. Entwicklungsmechn. Organ.*, 1913, xxxv, 595.

worms soon began to divide so that measurements of the original individuals became useless. It seemed best to consider all the new worms as still being a part of the worm from which they sprang. Hence to get the average size of a group, all the pieces of planarian in the dish were measured and the sum was divided by the original number of worms in that group. In considering the length one must bear in mind that the new worms produced by fission elongate by readjustment of substance to take on the typical form. This would tend to increase the length of planarian substance of worms which had divided often over those which had not. On the other hand, newly divided worms do not eat for some time. Those which have not divided are eating and growing in this interval. The two factors tend to counterbalance each other. Feeding began in October, and the dates given as final were taken in March. At the conclusion the worms in each dish were weighed after being dried with filter paper. The results are stated in Table I.

TABLE I.  
Series 1.

Group	Food	Original No.	Final No	Average increase.	Ratio of increase	Original total length	Final total length	Average increase	Ratio of increase	Final total weight	Final average weight.	Ratio of final weight.
						mm.	mm	mm		gm	gm.	
A. Small.	Liver..	27	40	0.48	1	141	356	7.9	1	0.159	0.00588	1
	Pituitary	27	73	1.70	3.54	116	514	14.7	1.86	0.197	0.00729	1.23
B. Medium.	Liver .	32	85	1.65	1	214.5	918.5	22	1	0.496	0.0155	1
	Pituitary	33	132	3.00	1.81	254	951.5	21	1.095	0.395	0.0119	0.76
C. Large.	Liver..	22	66	2.00	1	251	788	24.4	1	0.285	0.0129	1
	Pituitary	19	78	3.10	1.55	186	683.5	26.1	1.06	0.274	0.0144	1.11

It will be noted that the rate of fission in *Planaria* fed upon pituitary is in all cases decidedly larger than in the controls. The worms which were smallest in the beginning show the greatest effect, their rate of division being 3.54 times as great as that of the controls. The medium worms show an increase 1.81 times as great, and the largest worms 1.55 times as great as those fed

on liver. In length the medium and large pituitary worms developed almost equally with the controls. But the small worms increased 1.86 times as much in length as those fed on liver. Though the weights were not taken at the beginning of the experiment they must have been very nearly equal for the two divisions of each group, because the worms were equal in measurement and were in a starving condition at the time.

Here again we see a real difference in weight in favor of those fed upon pituitary in the case of the smallest worms. With the medium worms the controls are heavier. It should be remembered, however, that the worms fed upon liver have the appearance of being fatter than those eating pituitary. It seems that the liver diet leads to a storage of reserve material. If this is true, the weight of the liver-fed worms is greater than it should be, and any excess in favor of pituitary feeding, as shown in Group A, is more significant. During the time of this experiment the larger worms do not show a definite difference from the controls in length or weight. Judging, however, from the results obtained with small worms, these larger worms ought also to exceed the controls, were the time sufficient for the new individuals, produced by fission and subjected from inception to pituitary influence, to become adults; Group B is being continued with this idea in mind.

A second series was undertaken to demonstrate, if possible, differences in the effect of the various portions of the pituitary. Because of the uncertainty arising from fission in the first series and because of the greater effect noted in the younger individuals, very small worms were chosen for this experiment. They were almost all less than 4 mm. in length. Six groups nearly equal in size and number were formed. Group 1 was fed on pars glandularis. A portion well away from the cleft and in the center of the gland was used; this includes the cone of sinusoid vessels which pass from hilus to stem and contain large amounts of secretion. Group 2 was fed on cone substance. The cone is a structure appearing upon the pars intermedia in the ox pituitary.<sup>9</sup> In obtaining this food it was so cut that no pars intermedia was included with it. A large number of glands yield only a small

<sup>9</sup> Wulzen. R., *Anat. Rec.*, 1914, viii, 403.

amount of this substance. Group 3 was fed on pars intermedia cut carefully away in strips from pars posterior. Group 4 was given a portion of pars posterior from which pars intermedia was cut away. Group 5 had liver and Group 6 white matter of the brain. The feeding was continued until fission began to interfere with the results. After this no more food was given, but the dishes were allowed to stand so that the number of fissions might be observed. The data appear in Table II.

TABLE II.  
Series 2.

Group.	1. Pars glandularis		2. Cone.		3 Pars intermedia		4 Pars posterior		5. Liver.		6 White matter, brain.	
Date.	No	Average length	No	Average length	No	Average length	No	Average length	No.	Average length	No	Average length.
		mm		mm		mm		mm		mm		mm.
Nov. 12.....	48	3 1	49	2 9	50	2 8	47	3 0	46	3 0	47	3 0
" 22.....	48	3 7	42	3 6	48	3 8	47	4 0	44	3 5	47	3 5
Dec. 2.....	48	4 36	41	4 26	43	4 27	47	4 07	44	3 67	38	3 2
" 15.....	48	5 75	40	5 23	39	5 55	41	5 21	44	4 72	35	3 35
" 29.....	48	7 24	40	5 81	39	6 96	41	6 43	42	5 88	34	3 30
Jan. 19.....	48	7 82	38	6 44	39	7 11	41	6 56	42	6 21	25	2 96
Feb. 9.....	46	9 60	37	7 44	37	8 98	39	7 83	40	7 41	19	3 02
Total weight on Feb. 9, gm.....	0 174		0 077		0 124		0 109		0 116			
Average weight, gm.....	0 00378		0 00208		0 00335		0 00279		0 00290			
No. of new individuals on Mar. 13.	32		11		17		32		3			

The decreasing number of individuals during the experiment is due to the occasional loss of a worm in washing the dishes. But it expresses more than this in Group 6 where a harmful effect of the diet is certainly shown. In length and weight pars posterior, cone, and liver gave results so nearly equal that these portions of the pituitary may be called neutral as regards the growth of *Planaria*. It should be noted, however, that those fed on cone fall below in weight. The two remaining portions of the pituitary gave positive results. The worms fed upon pars

glandularis show an undoubted excess both in size and weight over the liver-fed controls. Those receiving pars intermedia have also increased in size and weight though not quite so much. The brain-fed worms which were intended as controls did not grow at all after the first 2 weeks. The varying values for their length are explained by the death of a considerable number of individuals. The meaning of this inhibition is unknown and will be investigated further. A striking result is to be noted in the number of new individuals produced by fission. No matter what the portion of pituitary fed, the rate of fission was greatly increased thereby. Pars glandularis and pars posterior gave a rate more than ten times as great as liver, while pars intermedia showed a rate almost six times, and cone substance one almost four times as great as liver.

#### DISCUSSION.

These experiments indicate positive effects on growth and reproduction when pituitary is fed to planarian worms. But they indicate further a separation in these effects. In Series 1, increased growth is shown in a pronounced way only by those worms which had received the diet from a very early period, while the effect on reproduction is marked no matter what the size (that is, age) of the worms when feeding was begun.

This result is one which reproduces a series of experiments of my own upon the growth of chicks. These experiments have not been previously reported by me as they needed the confirmatory evidence from other sources which now exists. In the first experiment I succeeded in raising two cocks from baby chicks to the age of 9 months. One received large amounts of fresh pars glandularis of ox pituitary, about  $\frac{1}{10}$  of the body weight, almost every day. The other had corresponding amounts of liver. During the early growth period the pituitary-fed chick showed inhibition of growth as did the chicks in my recorded experiments<sup>1</sup> and also those of Pearl.<sup>1</sup> However, when they became 4 months old, the control stopped growing rapidly while the pituitary cock continued its rapid growth and in 3 weeks had become larger and heavier than the control. This growth continued until the pituitary cock surpassed the control considerably. Its weight was then 1,882 gm. while that of the control was 1,597 gm. Its foot measured 206 mm. against 157 mm. of the control. This foot measurement exceeded that of any of the twelve cocks raised subsequently by me, presently to be reported. The resemblance between this result and that reported by Robertson<sup>3</sup> is remarkably close. Furthermore, the pituitary

cock began to crow at the age of 2 months, 2 weeks before the control, and throughout its life it crowed much oftener than did the control. The feathering of the pituitary cock was much handsomer, whiter, and silkier, and the feathers were longer and more downy than the control's.

In another series, twelve cocks, of the same breed as the above, were divided into two equal groups. Each cock in one group received about 15 gm. of fresh pars glandularis almost every day, while the others received the same amount of liver. They were 4 months old at the start and had not attained their full growth. The feeding continued for 3 months and at the end of that time no distinction could be made between the two groups as to size. But the crowing showed the same difference as before. It was counted for certain intervals of time in the middle of the day. During the major part of the experiment there were always many more crows given by the pituitary cocks than by the controls. Sometimes these would crow twenty times before the others crowed once. At the conclusion of the experiment they were crowing more equally, but it seemed as though all crowed more continually than do ordinary cocks. If the two lots could have been separated, the difference in number of crows would undoubtedly have been greater still and would probably have persisted to the end. The vigorous crows always started the others going and kept them at it.

Thus it seems with both fowl and *Planaria* that the feeding of pituitary must be begun in extreme youth and continued over prolonged periods in order that the effect on growth may appear. On the other hand, its effect upon reproduction in *Planaria* and the secondary sexual character of crowing in fowls is clearly defined even when feeding is begun with individuals well along toward the adult condition. The failure of pituitary feeding begun in the adolescent period to accelerate the growth of fowl might well be due to the advanced stage of ossification in the bones, but this cannot be true of the flat worms where there is no known anatomical difference between very large and very small individuals.

This perhaps indicates a distinction between the substance which produces growth and that which affects reproduction. The idea that some difference exists is strengthened by the second series of experiments with *Planaria*. These show that growth is accelerated by pars glandularis and pars intermedia but not at all by pars posterior, whereas rate of fission is increased by pars glandularis and pars posterior equally. It seems as though the substance which acts upon reproduction is stored everywhere in the gland and it is therefore able to show its effect when com-



paratively small quantities are eaten, but the growth substance, present in pars glandularis and pars intermedia, is quickly lost from the gland. Therefore to show its effect feeding must begin when tissues are young and very sensitive and must be continued over long periods of time.

This work gives a little indication as to the path of exit of secretion from the pituitary gland. From the evidence gathered by them, Herring,<sup>10</sup> Cushing and Goetsch,<sup>1</sup> and Cow<sup>11</sup> conclude that the substance secreted by pars intermedia leaves the gland through pars posterior and passes into the ventricle of the brain. But my studies with the pituitary of the ox, both gross and histological, have given me the impression that much more secretion must pass from the gland through the large blood vessels than can enter the brain. Thaon<sup>12</sup> has described and pictured blood vessels of pars glandularis gorged with secretion. I have repeated Thaon's work and have seen sinus after sinus packed with secretion of pars glandularis. The colloid substance present so often in the cleft of the ox pituitary has every appearance of being a product of pars intermedia. Blood vessels in the cleft region of pars glandularis, especially about the portion which is in contact with the cone, are very numerous and close to the surface and must offer admission to any substance present in the cleft. The path from pars intermedia to the blood sinuses of pars glandularis appears to offer much less resistance to the passage of secretion than that by way of pars posterior. If pars intermedia secretion passes largely through pars posterior, then pars posterior should incite growth as do pars glandularis and pars intermedia. But in this investigation upon *Planaria* it does not, nor has any one else reported such a result.

If we grant that pars intermedia sends its secretion mainly into pars glandularis, the question arises why pars posterior has so large an effect upon fission. If a guess might be made it would perhaps be this: The fresh secretion of the pituitary is produced by pars glandularis and pars intermedia, and most of it is carried quickly away by the blood vessels. This is the substance which is active in producing growth as well as in effecting

<sup>10</sup> Herring, P. T., *Quart. J. Exp. Physiol.*, 1903, i, 121.

<sup>11</sup> Cow, D., *J. Physiol.*, 1914-15, xlix, 375.

<sup>12</sup> Thaon, P., *Thèse de Paris*, 1907.

reproduction. All the secretion does not find its way into the blood vessels, however; some works out slowly through the posterior lobe. Moreover, there are in all portions of the gland blind alleys where masses of the secretion are brought to a standstill. As it lies in these cysts the character of the secretion changes. It loses its power over growth but can still affect sexual function. Thus pars posterior which has usually stored away much secretion can effect reproduction. It does not effect growth because no large amounts of fresh secretion are present in it.

I have attained positive effects upon fission with a diet of pars posterior while others have found nothing resulting but weakness and emaciation. This may be because *Planaria* are not injured by this substance so that in them it can produce its physiological effect. Apparently the higher animals are too severely harmed by this diet.

#### CONCLUSIONS.

1. The rate of fission in planarian worms is increased by a diet of pituitary substance, no matter what portion of the gland is used or what the age of the worms involved.

2. The growth of planarian worms is accelerated by a diet of pars glandularis and pars intermedia, provided that the worms are very small when the diet is begun.

3. There is indication of a distinction between the substance which produces fission and that which produces growth.

4. There is indication that the growth-producing substances of pars glandularis and pars intermedia leave the gland by way of the blood vessels of pars glandularis.



## EXPERIMENTAL STUDIES ON GROWTH.

### V. THE INFLUENCE OF CHOLESTEROL UPON THE GROWTH OF THE WHITE MOUSE.

By T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California, Berkeley.)

(Received for publication, May 25, 1916.)

#### *Previous Knowledge Concerning the Influence of Cholesterol upon Growth.*

Previous investigations in this laboratory<sup>1</sup> have shown that cholesterol, when administered subcutaneously in localities either near to or remote from the primary tumor, causes a very marked acceleration of the growth of Flexner-Jobling carcinoma in rats and also decidedly enhances the tendency of the primary tumor to give rise to metastases. The effect of cholesterol upon the growth of carcinomata is therefore analogous to that exerted by emulsions of the anterior lobe of the pituitary body<sup>2</sup> and by the growth-controlling principle, tethelin, which has been isolated from the anterior lobe of the pituitary body.<sup>3</sup>

It has also been shown that cholesterol, emulsified in hay infusion, causes a marked acceleration of the rate of multiplication of paramecia.<sup>4</sup>

The decided effects exerted by cholesterol upon the growth of carcinomata and the division rate of paramecia suggested the possibility that cholesterol, if added to the diet in unusual quantities, might have an appreciable effect upon the growth of mammals. The experiments which are about to be described were accordingly undertaken.

<sup>1</sup> Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, xvii, 344. Burnett, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1913, xi, 42; 1914, vii, 33.

<sup>2</sup> Robertson and Burnett, *J. Exp. Med.*, 1915, xvi, 280.

<sup>3</sup> Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 397, 409. Robertson and Burnett, *J. Exp. Med.*, 1916, xxiii, 631.

<sup>4</sup> Browder, A., *Univ. California Publications, Physiology*, 1915, v, 1.

*Description of the Experiments. Mode of Administering the Cholesterol. Dose Administered.*

Average healthy young mice, about 5 weeks of age, were taken at random from our stock, transferred to the cages described in the first article of this series,<sup>5</sup> and immediately, and thereafter until the age of 60 weeks, fed with cholesterol. Thirty-four males and thirty-five females were employed for this purpose. In every respect other than in the administration of the cholesterol these animals were handled and fed exactly as were the normal animals.<sup>6</sup> Their cages were placed in the same room as and not far removed from those containing the normal animals. They were only about 3 months younger than the normal animals and hence their life and growth were contemporary with the greater part of the life and growth of the normals. The cholesterol-fed animals and the normal animals were always weighed in the middle of the afternoon. Hence no possible factor other than the administration of cholesterol could account for the observed deviations of the growth of these animals from the normal.

The cholesterol employed was Merck's. The mixed whites and yolks of eggs were beaten and strained and the cholesterol, in the proportion of 1 gm. of cholesterol to 20 cc. of egg mixture, was rubbed up with the egg until it formed a smooth creamy emulsion. 5 cc. of this emulsion were placed in each compartment containing six mice, every day in the week except Sunday. The emulsion was eagerly eaten by the mice so that all of the cholesterol administered was consumed. The daily dose of cholesterol per mouse, added to that already present in the food, was therefore about 42 mg. The proportionate dose decreased with age, for it was not increased as the animals grew heavier. It was also somewhat larger for the females than for the males, since the females are somewhat less heavy than the males.

*Results.*—The weights of the cholesterol-fed and the normal animals are compared in Tables I and II and graphically illustrated in Figs. 1 and 2, the undotted curves representing the normal growth of white mice during the experimental period.

<sup>5</sup> Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 317.

<sup>6</sup> Robertson, *J. Biol. Chem.*, 1916, xxiv, 363.

the dotted curves the growth of the animals which received cholesterol. The dots represent the positions of the actual observations.

The effect of the administration of cholesterol is qualitatively the same in both sexes and quantitatively similar. Between the 5th and 10th weeks growth is very markedly retarded, so much so that at 10 weeks of age the cholesterol males lag 3.78 gm. and the females 3.84 gm. behind the normal animals. Between the 10th and 60th weeks, however, the growth of the cholesterol-fed animals is very decidedly accelerated, so that although they fail to catch up to the normals, yet at 20 weeks of age the cholesterol males lag only 2.73 gm. and the females 2.48 gm. behind the normals, the males having made up 1 gm. and the females 1.4 gm. of the defect due to the initial retardation. The subsequent

TABLE I  
*Cholesterol-Fed Male Mice*

Age.	Weight.		No weighed (Cholesterol-fed)	Age	Weight		No weighed. (Cholesterol-fed)
	Normal.	Cholesterol-fed.			Normal	Cholesterol-fed	
<i>wts.</i>	<i>gm.</i>	<i>gm</i>		<i>wts</i>	<i>gm.</i>	<i>gm</i>	
4	12.38		2	25	27 05	24 24	29
5	12.45	12 84	22	26	26 94	24 48	29
6	15.58	14 06	33	27	26 55	24 16	29
7	18.08	15 85	34	28	27 19	25 00	29
8	19.36	16 88	34	29	27 08	24 59	29
9	20 63	16 79	34	30	27 23	24 77	28
10	21.19	17 41	34	32	27 61	25 34	34
11	21.81	17 85	34	34	27 71	25 62	34
12	22.65	18 96	34	36	27 95	25 66	34
13	23 31	20 46	34	38	28 20	26 18	33
14	23 96	21 37	34	40	28 27	26 06	27
15	24.28	21 88	34	42	28 28	26 19	32
16	24.75	22 03	34	44	28 62	26 27	32
17	25 21	22 20	32	46	28 55	27 00	32
18	25.61	23 10	34	48	28 22	26 84	32
19	25 81	22 55	32	50	28 88	26 83	32
20	26 10	23 37	34	52	28 68	26 78	32
21	26 28	23 47	30	54	28 67	27 50	32
22	26.06	24 19	34	56	29 32	26 98	32
23	26.34	24 02	30	58	29 10	26 78	32
24	26 82	24 43	29	60	29 08	26 88	32

## Cholesterol-Fed Female Mice.

Age	Weight.		No. weighed. (Cholesterol-fed.)	Age.	Weight.		No. weighed. (Cholesterol-fed.)
	Normal.	Cholesterol-fed.			Normal.	Cholesterol-fed	
<i>wks</i>	<i>gm.</i>	<i>gm.</i>		<i>wks.</i>	<i>gm.</i>	<i>gm.</i>	
4	10.39		2	25	23.79	20.94	33
5	11.81	13.14	14	26	24.04	21.24	33
6	14.12	13.10	35	27	24.00	21.32	33
7	16.77	13.90	35	28	23.58	21.56	33
8	17.99	14.90	35	29	23.84	21.62	33
9	18.78	15.10	35	30	23.92	22.07	34
10	19.38	15.54	35	32	24.18	22.03	33
11	20.04	16.06	35	34	24.18	22.28	32
12	20.31	17.10	35	36	24.65	22.41	32
13	21.04	17.73	35	38	24.80	22.89	32
14	21.21	18.46	35	40	25.03	22.88	32
15	21.78	18.77	35	42	25.07	22.90	31
16	22.14	19.21	35	44	25.52	23.30	30
17	22.29	19.30	33	46	25.68	24.14	29
18	22.22	19.66	34	48	25.45	23.86	29
19	22.60	19.65	33	50	25.50	23.86	28
20	22.60	20.12	34	52	25.76	24.06	27
21	23.06	20.24	33	54	25.78	24.61	27
22	23.32	20.35	34	56	26.00	24.30	27
23	23.51	20.48	33	58	26.26	24.13	26
24	23.68	20.80	33	60	26.12	24.35	26

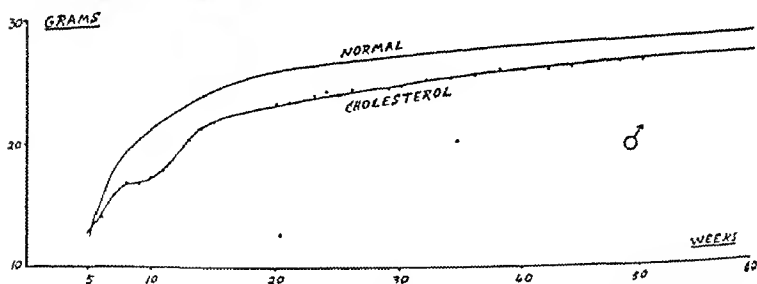
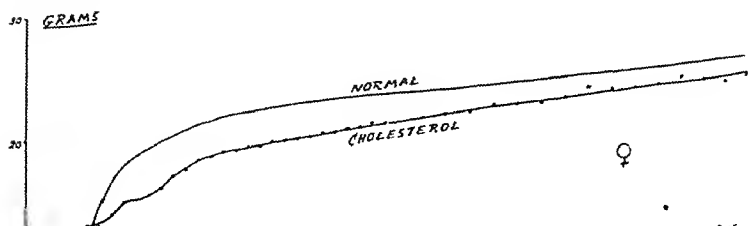


FIG. 1 Comparison of the growth curves of normal and of cholesterol-fed males



acceleration of growth is slight but consistent, so that the growth curves of the cholesterol-fed and normal animals from the 20th to the 60th weeks slowly but continuously approach one another. At 60 weeks of age the cholesterol-fed males only lag 2.20 gm. behind the normal males, having made up between the 20th and 60th weeks an additional 0.5 gm. of the defect due to the early retardation of growth, while the females lag 1.77 gm. behind the normals, having made up in the same time an additional 0.7 gm. of the defect. The initial retardation and subsequent acceleration of growth are so marked as to cause complete distortion of the growth curve, giving the appearance either of great prolongation and enlargement of the second growth cycle, as in the males, or of a new growth cycle interpolated between the second and third cycles in the normal curve, as in the females, the third growth cycle in both sexes being accelerated and curtailed. On comparing the growth curves of the cholesterol-fed animals with the growth curves of tethelin-fed animals<sup>7</sup> the close analogy between the two sets of growth curves cannot fail to be observed. It is evident that in their effect upon the third growth cycle of mice, as in their effect upon the growth of carcinomata, tethelin and cholesterol are analogous. In their potency these substances differ very greatly, however, for the effect upon the growth of mice which is brought about by the administration of a daily dose of 40 mg. of cholesterol is achieved by the daily administration of one-tenth of that amount of tethelin.

On comparing the variabilities of cholesterol-fed and normal animals (Tables III and IV and Figs. 3 and 4) a striking difference between the actions of cholesterol and of tethelin upon growth is revealed, for whereas tethelin causes a marked diminution in the variability of the animals to which it is fed, cholesterol gives rise to an equally marked increase. This is very clearly displayed in the case of the females (Fig. 4), and that the same effect was also exerted upon the variability of the males (Fig. 3) will be evident on observing that by chance the males selected for the experiment were initially (*i.e.*, at 5 weeks) much less variable than the average normals, the variability at 5 weeks of the males selected for the experiment being only 18.1 per cent

<sup>7</sup> Robertson, *J. Biol. Chem.*, 1916, xxiv, 397.



while the average variability of normal males at that age is 24.6 per cent. Yet between the 8th and 12th weeks the variability of the cholesterol-fed males exceeded that of the normals, and subsequent to 12 weeks the variability of the cholesterol-fed males remained very nearly equal to that of the normals, indicating that had the initial variability of the males selected for the experiment been normal, their variability after feeding them with cholesterol would have considerably exceeded that of the normals.

TABLE III.  
*Cholesterol-Fed Male Mice.*

Age	Variability.		Age.	Variability.	
	Normal.	Cholesterol-fed.		Normal.	Cholesterol-fed
<i>wks</i>	<i>per cent</i>	<i>per cent</i>	<i>wks.</i>	<i>per cent</i>	<i>per cent</i>
5	24.6	18.1	26	10.8	10.5
6	22.0	19.3	27	11.3	9.2
7	16.9	16.2	28	10.9	10.4
8	15.9	15.5	29	10.9	9.1
9	16.5	17.9	30	9.5	11.2
10	16.7	18.4	32	9.6	11.1
11	13.3	18.2	34	10.5	11.3
12	13.7	16.1	36	10.1	11.8
13	14.2	13.5	38	10.5	9.9
14	14.1	12.4	40	10.4	11.8
15	11.9	13.3	42	11.2	11.5
16	12.7	12.9	44	12.0	10.5
17	12.4	11.0	46	11.7	10.4
18	12.1	11.5	48	10.8	9.4
19	12.1	9.9	50	11.1	9.3
20	10.8	11.0	52	10.7	10.5
21	11.2	9.1	54	12.0	9.9
22	9.0	10.5	56	12.0	9.8
23	9.8	9.4	58	10.9	11.3
24	10.1	10.3	60	11.8	10.8
25	11.0	8.9			

TABLE IV.  
*Cholesterol-Fed Female Mice.*

Age.	Variability.		Age.	Variability.	
	Normal.	Cholesterol-fed		Normal.	Cholesterol-fed.
<i>u/s.</i>	<i>per cent</i>	<i>per cent</i>	<i>u/s.</i>	<i>per cent</i>	<i>per cent</i>
5	19.4	21.5	26	11.6	11.7
6	18.9	15.6	27	11.8	11.8
7	15.0	16.5	28	10.3	15.7
8	13.9	15.4	29	12.2	15.2
9	13.4	17.8	30	12.4	16.6
10	14.3	15.2	32	11.5	17.3
11	12.6	18.1	34	12.2	18.4
12	12.6	16.1	36	11.2	19.9
13	13.0	16.9	38	12.3	20.4
14	13.2	14.7	40	11.9	21.0
15	13.7	13.7	42	13.1	22.8
16	12.6	13.3	44	15.3	23.1
17	11.9	12.8	46	14.6	23.0
18	11.9	12.8	48	14.6	23.4
19	12.0	12.3	50	14.0	23.0
20	11.2	12.1	52	15.3	22.4
21	12.3	12.9	54	15.8	21.9
22	12.5	12.4	56	16.5	23.1
23	12.0	12.4	58	16.7	23.0
24	11.3	14.3	60	17.8	21.1
25	11.4	14.5			

PERCENT

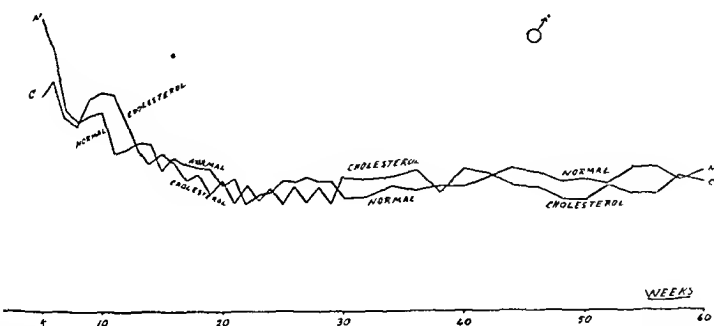


FIG. 3. Comparison of the variability curves of normal and of cholesterol-fed males.

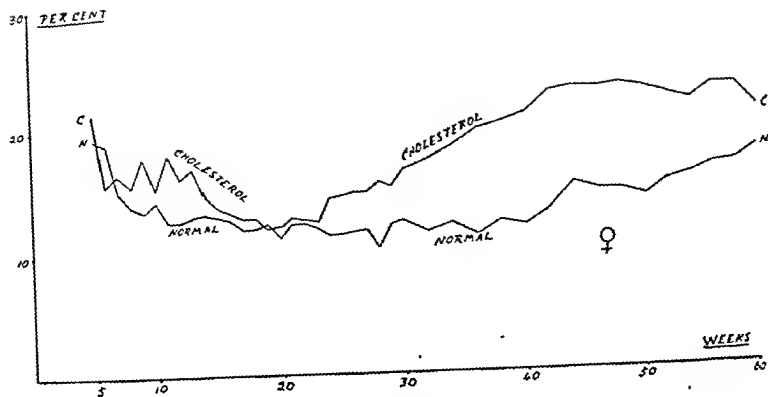


FIG. 4. Comparison of the variability curves of normal and of cholesterol-fed females.

The origin of the high variability of the cholesterol-fed animals lies in the fact that all of the animals do not display equal acceleration in the growth subsequent to 10 weeks of age. While the growth of some individuals is very greatly accelerated, producing, especially among the females, a group of very heavy animals, other individuals display little or no secondary acceleration of their growth. Since all of the animals are retarded in their growth during the first 5 weeks of the third growth cycle, this unequal acceleration in the latter part of the third growth cycle results finally in the production of a small group of very light animals, a larger group of animals of medium weight, and another small group of exceptionally heavy animals. In Fig. 5 female members of the extreme groups are compared. Both of the mice in the figure were 10 months old and had received since 5 weeks of age 40 mg. of cholesterol 6 days a week. The one on the left, however, weighed 14.5 gm. while that on the right weighed 44.5 gm. and 3 months later attained the weight of 48 gm.

#### *Differences in the External Appearance of the Cholesterol-Fed and the Normal Animals.*

Cholesterol is also analogous to tethelin in its effect upon the nutrition of the cutaneous tissues, for the coats of cholesterol-fed males are very noticeably superior in color and in smoothness

to those of normal males of the same age. This improvement of the coat, however, is not so striking as it is in the case of tethelin-fed animals. On the other hand the cholesterol-fed animals do not noticeably differ in the compactness of their build from normal animals of the same age, whereas tethelin-fed animals and animals fed upon the anterior lobe of the pituitary body, as I have pointed out in the articles alluded to above, differ very strikingly from normal animals in the superior compactness and heaviness of their build. The limbs and extremities of the tethelin- and pituitary-fed animals give the impression (although no



FIG. 5. Cholesterol-fed females, each 10 months of age, illustrating the high variability of cholesterol-fed animals.

actual measurements were made) of being thick and short and sturdy, while those of the cholesterol-fed animals are slender and delicate.

One very striking and invariable characteristic of cholesterol-fed males between the 5th and 10th weeks is the extreme prominence of the testicles. No actual measurements were made, but the impression was gained that while the body growth of cholesterol-fed males is strongly retarded, between the 5th and 10th weeks, the growth of the testicles is not similarly retarded, with the result that a striking disproportion between the size of the

animals and that of their testicles speedily becomes evident. Subsequent to the 10th week the cholesterol-fed males enter upon a period of rapid body growth and the disproportion in the size of the testicles disappears. This effect was observed in almost every male of the cholesterol-fed series, while it was not observed in any of the numerous experimental animals not receiving cholesterol.

*Absence of Deleterious Effects upon the Health of the Animals  
Attributable to the Administration of Cholesterol.*

It has been pointed out by Anitschkow,<sup>8</sup> Chalатов,<sup>9</sup> Bailey,<sup>10</sup> and others that the administration of cholesterol in daily doses of 0.2 to 0.8 gm. for periods of 50 to 100 days, or of equivalent amounts of cholesterol-containing foods such as brain or egg yolk, leads in rabbits to the formation of extensive deposits of anisotropic fat and ultimately to the formation of extensive lesions in the liver and other organs and to the formation of atheromata in the wall of the aorta, the formation of these lesions being accompanied by emaciation of the animal and ultimately leading to death. Anitschkow and Chalатов failed, however, to obtain similar lesions either in the liver or in the aorta in rats even after 5 months' feeding with a daily dose of one yolk of egg. Bailey<sup>11</sup> has similarly found that the daily administration of 0.1 to 0.5 gm. of cholesterol to guinea-pigs, even after 50 days, failed to produce atheromata in the aorta, although in some instances deposits of anisotropic fat were found in the liver and adrenals.

In the present experiment no deleterious effects which could be attributed to the feeding of cholesterol were ever displayed by any of the animals. Although the dose of cholesterol administered was a very large one, about 1.6 gm. per kilo of body weight 6 days per week, no evidence of the formation of serious lesions has been obtained. One male which had received this dose of

<sup>8</sup> Anitschkow, N., *Beitr. path. Anat. u. allg. Path.*, 1913, lvi, 379; 1914, lvii, 201.

<sup>9</sup> Chalатов, S. S., *Arch. path. Anat.*, 1912, ccvii, 452; *Beitr. path. Anat. u. allg. Path.*, 1914, lvii, 85.

<sup>10</sup> Bailey, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1914, xii, 68.

<sup>11</sup> Bailey, *Proc. Soc. Exp. Biol. and Med.*, 1915, xiii, 60.

cholesterol for 50 days was examined by Dr. G. Y. Rusk, who was unable to find any lesions in the wall of the aorta, while one female which had received the above dose of cholesterol for 463 days and another which had received it for 511 days were examined by Dr. Bailey who also failed to find any evidence of aortic lesions although deposits of anisotropic fat were found in the liver. That no other acute conditions were caused by the cholesterol feeding may be inferred from the fact that the cholesterol-fed animals displayed normal health throughout the experiment and the percentage of deaths to the date of writing (the animals being about 17 months of age) has not differed appreciably from the percentage of deaths occurring in a like period of time among normal animals.

It is evident, therefore, that the ability of the tissues to dispose adequately of excess of cholesterol varies in different species of animals and is very slight in the rabbit as compared with the guinea-pig, rat, and mouse. That this greater tolerance displayed by mice is not attributable to non-absorption of the cholesterol administered is evident from the very striking effects upon the growth of the animals to which, as we have seen, the administration of cholesterol leads.

### *Analysis of the Causes of Death.*

Between the 10th and 60th weeks two deaths were recorded among the cholesterol-fed males and eight among the cholesterol-fed females. The following is a summary of the causes of death:

#### Males.

Pneumonia.....	1
Unidentified cause .....	1

#### Females.

Cancer.....	5
Paratyphoid.....	1
Pneumonia.....	1
Unidentified cause.....	1

## SUMMARY.

1. The administration of 40 mg. per day per animal of cholesterol to mice, beginning at 5 weeks after birth (conclusion of the second growth cycle), leads to marked retardation of growth during the earlier portion of the third growth cycle, between the 5th and 10th weeks. From the 10th week onward, however, growth is decidedly accelerated, although the acceleration is insufficient to compensate entirely for the initial retardation.

2. The influence of cholesterol upon the third growth cycle in mice is therefore comparable with that produced by the administration of much smaller doses of tethelin.

3. The influence of cholesterol upon the variability of the animals to which it is fed is, however, directly opposite to the effect exerted by tethelin, for while tethelin reduces the variability of the animals to which it is fed, cholesterol increases their variability. This increase in variability is mainly attributable to the very unequal acceleration, in different individuals, of the latter portion of the third growth cycle.

4. Cholesterol-fed animals do not noticeably differ in build from normal animals. Their coats are smoother and more glossy in appearance than those of normal animals of the same age.

5. Between the 5th and 10th weeks the testicles of cholesterol-fed males becomes very prominent. This disproportion between the size of the testicles and that of the animals bearing them rapidly disappears subsequent to the 10th week. It is probably due to absence of retardation of the growth of the testicles during the first 5 weeks of feeding, so that the retardation of the body growth of the animals leads to disproportionate size of the testicles. The subsequent acceleration of body growth restores the normal proportion.

6. No deleterious effects attributable to the feeding of cholesterol were observed. Three animals which were examined after 50, 463, and 511 days of feeding respectively showed no evidence of lesions in the wall of the aorta such as are observed in rabbits after feeding relatively smaller doses for comparatively brief periods of time.

## EXPERIMENTAL STUDIES ON GROWTH.

### VI. THE INFLUENCE OF LECITHIN UPON THE GROWTH OF THE WHITE MOUSE.

By T. BRAILSFORD ROBERTSON.

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California, Berkeley.)

(Received for publication, May 25, 1916.)

#### *Previous Knowledge Concerning the Influence of Lecithin upon Growth.*

Previous investigations have shown that the effect of lecithin upon growth depends very considerably upon the type and stage of development of the tissue treated. On the one hand King<sup>1</sup> and Johnson<sup>2</sup> found that frog embryos, when fed with egg lecithin grow more rapidly, both in size and in weight, than normal tadpoles, and Bain<sup>3</sup> found that the subcutaneous administration of lecithin leads to a leukocytosis and an increase in the number of red corpuscles, facts which may possibly indicate an increased rate of formation of these tissues. On the other hand Johnson<sup>2</sup> found that lecithin, when administered by mouth, inhibits pigment formation in tadpoles, Browder<sup>4</sup> has shown that the presence of egg lecithin in the culture medium retards the division rate of paramecia, and I have found<sup>5</sup> that egg lecithin, when added to sea water, strongly retards the earlier stages in the development of fertilized sea urchin eggs.

Previous investigations in this laboratory<sup>6</sup> have also shown that lecithin, prepared from egg yolks by extracting them with ether and precipitating with acetone, causes a distinct retarda-

<sup>1</sup> King, H. D., *Biol. Bull.*, 1907, xiii, 40.

<sup>2</sup> Johnson, M., *Univ. California Publications, Zoology*, 1913, xi, 53.

<sup>3</sup> Bain, W., *Lancet*, 1912, i, 918.

<sup>4</sup> Browder, A., *Univ. California Publications, Physiology*, 1915, v, 1.

<sup>5</sup> Robertson, T. B., *Arch. Entwicklungsmech. Organ.*, 1913, xxxvii, 497.

<sup>6</sup> Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, xvii, 344.



tion of the growth of Flexner-Jobling carcinoma in rats, when administered subcutaneously in the neighborhood of the tumor. Lecithin which has been prepared in this manner contains a very considerable proportion of cephalin<sup>7</sup> and possibly a certain admixture of other phospholipins to which the observed effects might conceivably be attributable. We have also found,<sup>8</sup> however, that the lecithin prepared by extracting the anterior lobe of the pituitary body with alcohol, removing the tethelin<sup>9</sup> contained in this extract by precipitation with ether, evaporating the resultant mixture to dryness, taking up the residue in ether, and precipitating with acetone also leads, when administered subcutaneously in localities remote from the tumor, to a decided retardation of the growth of Flexner-Jobling carcinoma. Since cephalin is insoluble in alcohol the lecithin thus prepared must have been free from admixture with cephalin. It is probable, therefore, that the retarding effect of egg lecithin upon the growth of carcinomata is at least in part, due to lecithin itself.

*Description of the Experiments. Methods of Preparing and Administering the Lecithin. Doses Administered.*

Mice about 5 weeks of age were taken at random from our stock transferred to the cages described in the first article of this series, and immediately and thereafter during the entire period of the experiment, fed with lecithin. Thirty-six males and thirty-six females were fed with egg lecithin and twenty-four males with pituitary lecithin. In every respect other than in the administration of the lecithin these animals were handled and fed exactly as were the normal animals.<sup>10</sup> The animals fed with egg lecithin were about 1 month, and those fed with pituitary lecithin about 4 months younger than the normals so that their life and growth were contemporary with the greater part of the life and growth of the normals. The lecithin-fed animals and the normal animals were always weighed in the middle of the afternoon. Unfortunately the average weight of the group of animals taken at random to be fed with egg lecithin was considerably below the

<sup>7</sup> Steadman and Thierfelder, H., *Z. physiol. Chem.*, 1907, liii, 370.

<sup>8</sup> Robertson and Burnett, *J. Exp. Med.*, 1916, xxiii, 631.

<sup>9</sup> Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347.

<sup>10</sup> Robertson *J. Biol. Chem.*, 1916, xxiv, 362.

average weight of normals, the mean defect at 4 and 5 weeks of age being 1.2 gm. in the males and 1 gm. in the females. The reason for this lies in the extremely high variability of normal animals at the age when experimental animals were selected, rendering it not improbable that a limited number of animals chosen at random may deviate somewhat widely from the true average in weight. Thus the variability of the weight of male mice at 4 or 5 weeks of age is over 24 per cent while the mean weight is 12.4 gm.; hence one animal out of every three may be expected to deviate from the average weight by as much as 3 gm. Similarly one female out of every three may be expected to deviate from the average weight by over 2 gm.<sup>11</sup> The abnormality of the animals employed was unfortunately not detected until a late stage in the investigation and it will be necessary, in order to be certain of the validity of the results obtained, to repeat the experiment with a more representative group of animals. However, the results obtained with egg lecithin, in so far as it is possible to interpret them in view of the above facts, are confirmed, as will be seen, by the results obtained with pituitary lecithin and they are therefore tentatively submitted at this time subject to reconsideration in the light of results obtained with a more representative group of animals. Incidentally the difficulty of accurately comparing the growth of these animals with that of normal animals reveals the necessity of ascertaining, before undertaking *comparative* experiments upon growth, that the limited number of animals chosen for the experiment are truly representative of the average.

The animals selected for feeding with pituitary lecithin were slightly, but not to any important extent, supernormal in weight.

The egg lecithin was prepared in the following manner: To the yolk of eggs was added an equal volume of 10 per cent sodium chloride solution and the mixture was well shaken. This mixture was then shaken with an equal volume of ether until it became of a vaseline-like consistency. On standing, in the course of 24 to 48 hours, the ether and water layers separate and the ether may then be pipetted or syphoned off. This ether extract was evaporated, in an open vessel at room temperature, to about one-

<sup>11</sup> Robertson, *J. Biol. Chem.*, 1916, xxiv, 409.

third of its volume, two volumes of acetone were added, and the precipitate was collected, washed once in acetone, drained, heated for a brief period to drive off the major part of the acetone, and then dried at room temperature over sulfuric acid. As stated above, lecithin prepared in this manner contains a considerable proportion of cephalin and possibly other phospholipoids.

6 gm. of this preparation were placed in 60 cc. of the mixed white and yolk of egg and the mixture was allowed to stand on ice over night. At the end of this period the mass of lecithin had attained a soft jelly-like consistency and was easily rubbed up into a uniform emulsion with the egg. 5 cc. of this mixture were supplied daily, except Sundays, to every six mice, so that the daily dose, assuming the material to have been completely consumed, was 83 mg. As a rule the mice of all experimental classes eagerly consumed the egg mixtures supplied to them, but the mice fed with the mixture just described constituted an exception to the rule. They appeared distinctly averse to the mixture and it was only slowly consumed, although in the course of 24 hours, as a rule, but little of it remained uneaten.

The pituitary lecithin was prepared from the supernatant fluid after the precipitation of tethelin from the alcoholic extract of the anterior lobe of the pituitary body.<sup>11</sup> The fluid was evaporated, without exposure to air, until the boiling point reached 78°C. It was then cooled to 0°C. for 24 to 48 hours, which resulted in the deposition of a small amount of material, probably including the greater part of any unprecipitated tethelin, since tethelin is only sparingly soluble in ice-cold alcohol. The fluid decanted from this deposit was then evaporated, without exposure to air, the residue taken up in a small amount of ether, and several volumes of acetone were added to the resultant solution. The precipitate was collected on a hardened filter, washed in acetone, and dried at room temperature over sulfuric acid. The yield was about 20 mg. per anterior lobe or 1.33 per cent of the fresh tissue. 1 gm. of this material was rubbed up in 20 cc. of distilled water, forming a viscous emulsion, and 2 cc. of this were mixed with 20 cc. of egg yolk and white mixture which was divided equally among the twenty-four mice daily, except Sundays. The daily dose was therefore about 4 mg. per animal. This mixture was always quickly and completely consumed.

According to Parke<sup>12</sup> lecithin forms 10.7 per cent of the egg yolk. Assuming the egg yolk to be half the volume of the white, the mixture of white and yolk which was fed to normal animals, and to which the above mentioned quantities of lecithin were added before supplying it to the experimental animals, contained 3.6 per cent of lecithin; that is, 30 mg. per animal per day. The pituitary lecithin added to the egg mixture constituted, therefore, only a trifling addition to the lecithin already present.

*Results.*—The weights of the animals fed with egg lecithin and those of the normal animals are compared in Tables I and II and graphically illustrated in Figs. 1 and 2, the smooth curves representing the normal growth of white mice during the experimental period and the dotted curves the growth of the animals

TABLE I.  
*Lecithin-Fed Male Mice.*

Age.	Weight.		No. weighed. (Lecithin-fed.)	Age.	Weight.		No. weighed. (Lecithin-fed.)
	Normal.	Lecithin-fed.			Normal.	Lecithin-fed.	
<i>wks.</i>	<i>gm.</i>	<i>gm.</i>		<i>wks.</i>	<i>gm.</i>	<i>gm.</i>	
4	12.35	10.86	18	25	27.05	24.50	34
5	12.45	11.53	36	26	26.94	25.10	36
6	15.58	13.11	36	27	26.55	24.97	34
7	18.08	15.07	36	28	27.19	25.26	35
8	19.36	16.56	36	29	27.08	25.27	33
9	20.63	17.69	36	30	27.23	25.74	34
10	21.19	19.13	36	32	27.61	26.04	36
11	21.81	20.06	36	34	27.71	26.32	36
12	22.65	20.50	36	36	27.95	26.86	35
13	23.31	21.10	36	38	28.20	26.84	35
14	23.96	21.49	36	40	28.27	27.02	33
15	24.28	22.17	36	42	28.28	27.23	33
16	24.75	22.22	36	44	28.62	27.15	33
17	25.21	22.60	36	46	28.55	27.17	33
18	25.61	23.25	36	48	28.22	27.47	32
19	25.81	23.72	36	50	28.88	27.44	31
20	26.10	24.04	36	52	28.68	27.76	31
21	26.28	24.17	36	54	28.67	28.07	30
22	26.06	24.86	36	56	29.32	27.82	30
23	26.34	24.57	36	58	29.10	27.93	30
24	26.82	24.43	36	60	29.08	27.75	28

<sup>12</sup> Parke, J. L., *Med.-Chem. Untersuch.*, 1866, i, 209.

TABLE II.  
*Lecithin-Fed Female Mice.*

Age	Weight		No weighed (Lecithin-fed)	Age	Weight.		No weighed (Lecithin-fed)
	Normal	Lecithin-fed.			Normal.	Lecithin-fed.	
u/s	gm	gm.		u/s.	gm.	gm	
4	10 39	8 87	15	25	23 79	21 34	35
5	11 81	11 42	31	26	24 04	21 66	35
6	14 12	13 35	36	27	24 00	21.61	35
7	16 77	15 04	36	28	23.58	21 92	33
8	17 99	16 47	36	29	23 84	22.06	35
9	18 78	17 00	36	30	23.92	22.34	29
10	19 38	17 32	36	32	24.18	22 29	34
11	20 04	18 08	36	34	24 18	22 56	33
12	20 31	18 67	36	36	24.65	22.88	33
13	21 04	18 71	35	38	24 80	22.82	33
14	21 21	19 26	35	40	25 03	22 97	33
15	21 78	19 71	35	42	25.07	23.00	33
16	22 14	20 03	35	44	25 52	23.29	33
17	22 29	20 26	35	46	25.68	23 20	32
18	22 22	20 50	35	48	25.45	24.02	32
19	22 60	20 77	35	50	25 50	24 08	31
20	22 60	20 64	35	52	25.76	24 60	31
21	23 06	21 03	35	54	25 78	24 55	29
22	23 32	21 13	35	56	26.00	24 28	29
23	23 51	21 31	35	58	26.26	24 69	29
24	23 68	21 27	35	60	26.12	24 09	28

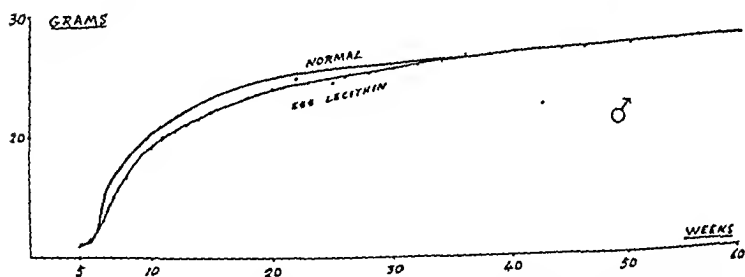


FIG. 1. Comparison of the growth curves of normal males and males fed with lecithin derived from egg yolk. Ordinates of normal curve diminished by 1.2 gm.

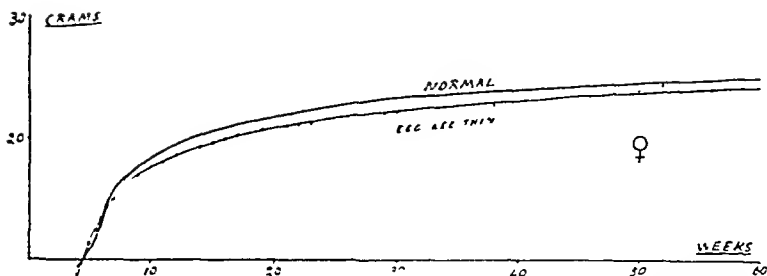


FIG. 2 Comparison of the growth curves of normal females and females fed with lecithin derived from egg yolk. Ordinates of normal curve diminished by 1 gm.

which received lecithin. The dots represent the positions of the actual observations. The ordinates of the normal curves are reduced by 1.2 gm. in the case of the males and 1.0 gm. in the case of the females in order, as far as possible, to compensate for the initial subnormality of the lecithin-fed animals and facilitate a comparison of the two curves.

The effect of the administration of egg lecithin upon the form and proportions of the growth curve is evidently extremely small; a fact which, in view of the very large dosage, is rather surprising. Of course it is uncertain to what extent a uniform reduction of the ordinates of the normal curve is a valid method of representing the normal growth curve of initially subnormal animals; but if it be assumed that this procedure may yield an approximation to the normal curve in such cases, then the only effect of the administration would appear to consist in a very slight—in the case of the males a barely perceptible—retardation of growth without deformation of the curve, i.e., a retardation which is nearly uniform throughout the whole of the third cycle. There appears to be some slight acceleration in the latter part of the cycle in the males, but as this is not evidenced in the females it is probably apparent and not real, resulting from the initial abnormality of the experimental animals.

The variabilities of normal animals and of those fed with egg lecithin are compared in Tables III and IV and Figs 3 and 4. Here again we see very little effect of the administration when the necessary allowance is made for initial differences.

variabilities of the normal and the experimental groups of animals. The lecithin-fed animals, being initially lighter than the normals, were also initially less variable.<sup>13</sup> When the initial point on the variability curve of the lecithin-fed animals is raised to the level of the initial point on the variability curve of the normal animals it is seen that the administration of egg lecithin caused little if any alteration in the variability of the males and a slight increase in the variability of the females from the 30th week onwards, an effect which, again, may be apparent and not real.

TABLE III.  
*Lecithin-Fed Male Mice.*

Age	Variability.		Age.	Variability.	
	Normal.	Lecithin-fed.		Normal.	Lecithin-fed.
<i>wks</i>	<i>per cent</i>	<i>per cent</i>	<i>wks.</i>	<i>per cent</i>	<i>per cent</i>
4	24.4	23.1	25	11.0	8.6
5	24.6	23.2	26	10.8	8.4
6	22.0	21.7	27	11.3	7.4
7	16.9	19.4	28	10.9	8.8
8	15.9	18.5	29	10.9	8.8
9	16.5	15.8	30	9.5	8.8
10	16.7	13.7	32	9.6	8.8
11	13.3	11.4	34	10.5	8.5
12	13.7	10.2	36	10.1	8.8
13	14.2	9.8	38	10.5	8.8
14	14.1	9.4	40	10.4	8.3
15	11.9	9.9	42	11.2	9.5
16	12.7	8.9	44	12.0	10.0
17	12.4	8.5	46	11.7	10.1
18	12.1	8.7	48	10.8	9.1
19	12.1	7.5	50	11.1	9.7
20	10.8	8.0	52	10.7	9.3
21	11.2	8.2	54	12.0	8.7
22	9.0	7.6	56	12.0	9.0
23	9.8	7.9	58	10.9	7.8
24	10.1	8.7	60	11.8	8.1

<sup>13</sup> Robertson, *J. Biol. Chem.*, 1916, xxiv, 372, 392, 405.

TABLE IV  
*Lecithin-Fed Female Mice.*

Age	Variability		Age	Variability	
	Normal	Lecithin fed		Normal	Lecithin-fed
<i>weeks</i>	<i>per cent</i>	<i>per cent</i>	<i>weeks</i>	<i>per cent</i>	<i>per cent</i>
4	23.1	16.3	25	11.4	9.1
5	19.4	15.8	26	11.6	8.9
6	18.9	12.7	27	11.8	9.8
7	15.0	9.7	28	10.3	9.8
8	13.9	8.8	29	12.2	10.2
9	13.1	8.6	30	12.1	10.6
10	11.3	9.6	32	11.5	11.2
11	12.6	8.2	34	12.2	10.9
12	12.6	8.6	36	11.2	10.8
13	13.0	8.2	38	12.3	11.4
14	13.2	7.6	40	11.9	11.2
15	13.7	8.9	42	13.1	12.1
16	12.6	8.4	44	13.3	12.9
17	11.9	8.5	46	14.6	13.6
18	11.9	9.0	48	14.6	14.2
19	12.0	8.6	50	14.0	14.1
20	11.2	8.5	52	15.3	14.0
21	12.3	8.8	54	15.8	14.7
22	12.5	9.1	56	16.5	14.1
23	12.0	9.0	58	16.7	13.5
24	11.3	9.4	60	17.8	13.1

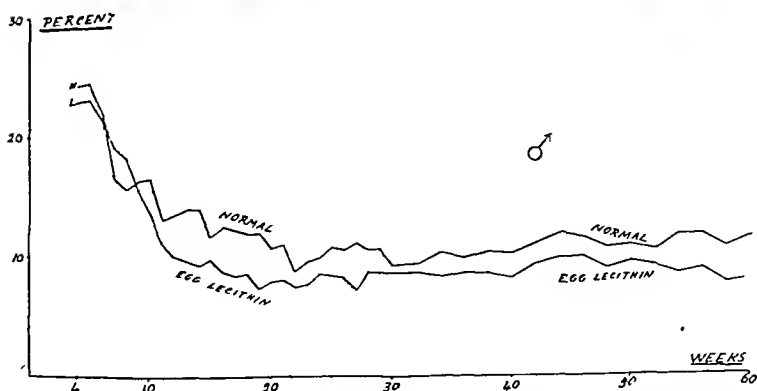


FIG. 3. Comparison of the variability curves of normal males and males fed with lecithin derived from egg yolk



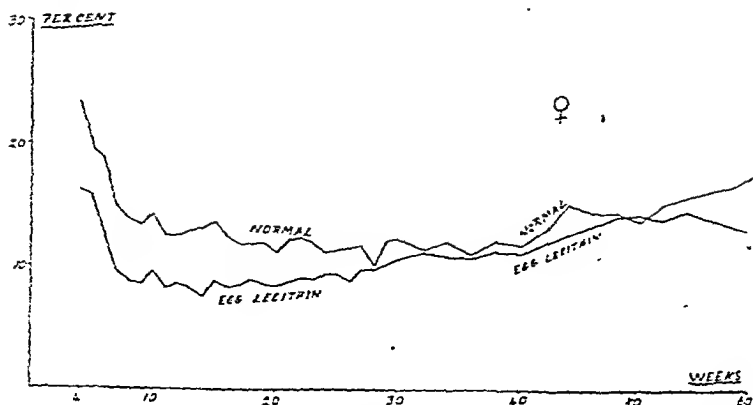


FIG. 4. Comparison of the variability curves of normal females and females fed with lecithin derived from egg yolk.

The weights of the animals fed with pituitary lecithin and those of normal animals are compared in Table V and graphically illustrated in Fig. 5. It will be seen that the lecithin-fed animals were initially slightly supernormal in weight but that within 2 weeks of commencing the feeding of lecithin they dropped slightly behind the normals in weight, remaining slightly and nearly uniformly subnormal in weight throughout the experiment. Apparently the administration of pituitary lecithin caused a slight and nearly uniform retardation of weight and no acceleration, unless the slight deviations from the smoothed curve at 10 weeks indicate some acceleration. These deviations are so slight, however, that no certain conclusions can be drawn from them. We may infer, therefore, that pituitary lecithin causes a slight retardation of growth in the third growth cycle and no appreciable acceleration at any period during the cycle.

TABLE V  
*Male Mice Fed with Pituitary Lecithin.*

Age	Weight		No weighed (Lecithin-fed)	Age	Weight		No weighed (Lecithin-fed)
	Normal	Lecithin-fed			Normal	Lecithin-fed	
wt. s.	gm.	gm.		wt. s.	gm.	gm.	
5	12.45	14.63	15	26	26.91	25.89	22
6	15.58	15.51	21	27	26.55	25.70	23
7	18.08	16.77	21	28	27.19	26.14	22
8	19.36	17.92	21	29	27.08	26.09	23
9	20.63	17.92	21	30	27.23	26.06	18
10	21.19	19.10	21	32	27.61	26.72	23
11	21.81	19.06	21	34	27.71	26.48	23
12	22.65	20.16	21	36	27.95	26.54	23
13	23.31	21.38	21	38	28.20	26.91	23
14	23.96	22.58	21	40	28.27	27.17	23
15	24.28	23.23	21	42	28.28	27.76	23
16	24.75	23.73	24	44	28.62	27.85	23
17	25.21	24.23	21	46	28.55	27.85	23
18	25.61	24.48	23	48	28.22	28.11	22
19	25.81	24.74	24	50	28.88	28.00	22
20	26.10	25.15	23	52	28.68	28.02	22
21	26.28	25.04	23	54	28.67	27.84	22
22	26.06	25.61	23	56	29.32	27.85	20
23	26.34	25.35	23	58	29.10	28.00	16
24	26.82	25.65	23	60	29.08	28.19	16
25	27.05	26.02	23				

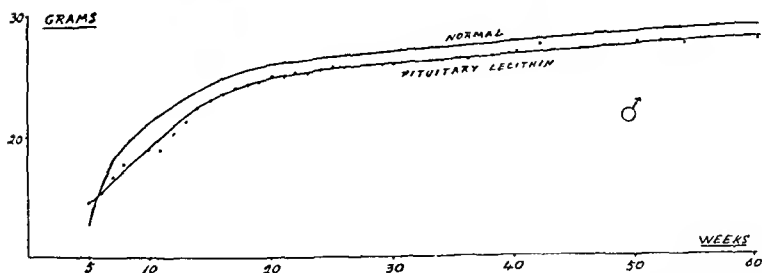


FIG. 5. Comparison of the growth curves of normal males and males fed with lecithin derived from the anterior lobe of the pituitary body.

The variabilities of the animals fed with pituitary lecithin and those of normals are compared in Table VI and Fig. 6. It will be seen that the administration of pituitary lecithin caused an evident diminution of variability.

TABLE VI.  
*Male Mice Fed with Pituitary Lecithin.*

Age.	Variability.		Age.	Variability.	
	Normal.	Lecithin-fed.		Normal.	Lecithin-fed.
wks.	per cent	per cent	wks.	per cent	per cent
5	24.6	24.6	26	10.8	7.2
6	22.0	19.7	27	11.3	7.4
7	16.9	17.1	28	10.9	7.1
8	15.9	13.9	29	10.9	7.4
9	16.5	15.1	30	9.5	7.3
10	16.7	12.6	32	9.6	7.4
11	13.3	13.4	34	10.5	7.6
12	13.7	13.7	36	10.1	7.5
13	14.2	12.3	38	10.5	7.5
14	14.1	10.8	40	10.4	7.8
15	11.9	9.2	42	11.2	7.5
16	12.7	8.8	44	12.0	7.6
17	12.4	7.8	46	11.7	7.5
18	12.1	7.3	48	10.8	7.3
19	12.1	8.3	50	11.1	7.9
20	10.8	6.9	52	10.7	7.9
21	11.2	7.2	54	12.0	7.6
22	9.0	6.8	56	12.0	8.6
23	9.8	6.8	58	10.9	7.6
24	10.1	7.0	60	11.8	7.7
25	11.0	6.4			

Considering the much smaller dose administered, pituitary lecithin would appear to exert a considerably greater effect upon the growth of mice than egg lecithin. Whether this disparity of effect is attributable to the initial subnormality of the animals fed with the egg lecithin, or to an admixture of other and more potent phospholipoids with the pituitary lecithin it is impossible at present to assert. Having regard, however, to the fact that the pituitary lecithin comprised only a small proportion of the lecithin present in the egg mixture which was administered to the animals, even the slight effects observed are probably to be

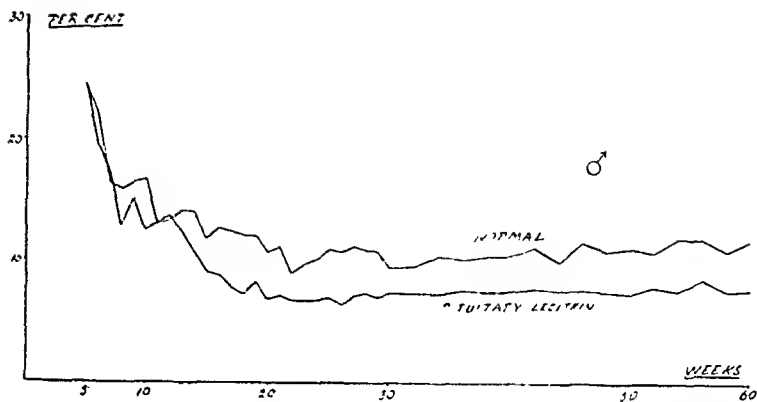


FIG. 6. Comparison of the variability curves of normal males and males fed with lecithin derived from the anterior lobe of the pituitary body.

attributed to admixture of some other substance, possibly a trace of tethelin, or at all events to some peculiarity of lecithin prepared from the anterior lobe of the pituitary body.

Summarising the results, it is evident that the effect of lecithin upon the growth of white mice is much less than might be expected from the very decided action exerted by lecithin upon the growth of carcinomata, the divisions of paramecia, and the growth of tadpoles. The reason for the lack of action when administered by mouth to mice probably lies in the fact that lecithin is very readily split by lipase and is therefore, in all probability, not absorbed as such to any appreciable extent. In the experiments on carcinoma which I have cited the lecithin was administered subcutaneously and was therefore for some time present in the tissues as such, and the absorption by paramecia or tadpoles may not improbably differ from absorption in the alimentary canal of a mammal. The observed retarding action may possibly be attributable to the injurious effects of the absorption of excess of choline.

*Differences in the External Appearance of the Lecithin-Fed and the Normal Animals.*

No very evident external differences were observed between the normal and the lecithin-fed animals, excepting that the coats of the animals fed with egg lecithin presented from an early age a wretched appearance. This was possibly in part due to the slow consumption of the daily supply of egg. The animals thus had ample opportunity to get the egg upon their coats, and as the mixture was extremely sticky many of the animals constantly had small patches of dried egg-lecithin mixture adhering to their coats. No such effect upon the coat was observed among the animals fed with pituitary lecithin.

All of the animals fed with lecithin displayed an unusually high degree of susceptibility to infection. Among the males fed with egg lecithin seriously infected wounds were common, resulting from injuries received in fighting, while among the animals fed with pituitary lecithin a form of infection of the respiratory tract was met with, accompanied by sneezing, gurgling noises in the throat, and progressive slow loss of weight. No similar cases were encountered in any of the other experimental groups, but it repeatedly appeared in this group subsequent to the 50th week, despite the fact that animals suspected of infection were isolated until their condition became evident and were then killed, and the food vessels, nests, and cage were repeatedly washed with alcohol.

*Analysis of the Causes of Death.*

Between the 10th and the 60th weeks seven deaths were recorded among the males and seven among the females which were fed with egg lecithin. During the same period eight deaths were recorded among the animals which were fed with pituitary lecithin. The following is a summary of the causes of death:

*Males fed with egg lecithin.*

Cancer . . . . .	1
Gastric ulcer . . . . .	1
Infected wounds received while fighting.....	4
Unidentified cause .. . . .	1

## Females fed with egg lecithin.

Cancer.....	1
Duodenal ulcer.....	1
Abscess.....	1
Infected injury.....	1
Acute nephritis.....	1
Unidentified causes.....	2

## Males fed with pituitary lecithin.

Unidentified infection of respiratory tract <sup>14</sup> .....	7
Unidentified cause.....	1

## SUMMARY.

1. The administration by mouth of 80 mg. per day per animal of egg lecithin, beginning at 4 weeks after birth (conclusion of the second growth cycle), leads to no deformation of the curve of growth, the only demonstrable effects of the administration consisting in a very slight uniform retardation of growth and a low degree of resistance to infection, both effects being not improbably attributable to the injurious action of excess of choline absorbed from the alimentary tract.

2. The administration by mouth of 4 mg. per day of lecithin derived from the anterior lobe of the pituitary body produces similar effects. Having regard to the comparatively small dose administered it is possible that these effects may in part have been due to admixture of other and more potent substances with lecithin derived from this source or at all events to a peculiarity of lecithin derived from the anterior lobe of the pituitary body.

3. The lack of effect of lecithin administered by mouth in comparison with its effects when administered subcutaneously or to lower organisms is probably attributable to the fact that lecithin is completely split during digestion and is not absorbed to any appreciable extent as such.

<sup>14</sup> One of these animals, in postmortem examination, was found to be suffering from cancer.



## EXPERIMENTAL STUDIES ON GROWTH.

### VII. THE INFLUENCE OF THE ADMINISTRATION OF EGG LECITHIN AND OF CHOLESTEROL TO THE MOTHER, UPON THE GROWTH OF SUCKLING MICE.

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(Received for publication May 25, 1916)

#### *Object of the Experiments*

While lecithin has been shown to exert a retarding action upon certain types of growth, such as the early stages of the development of sea urchin embryos,<sup>1</sup> the growth of carcinoma,<sup>2</sup> and the division rate of paramecia,<sup>3</sup> it has also been found to accelerate certain other types of growth, such as that leading to the production of leukocytes and erythrocytes<sup>4</sup> and the development of tadpoles.<sup>5</sup> Similarly it has been found that cholesterol, while very markedly retarding the initial portions of the third growth cycle in mice, nevertheless accelerates the later growth of mice,<sup>6</sup> also the growth of carcinomata<sup>7</sup> and the division rate of paramecia.<sup>3</sup> It is evident therefore that these substances may exert an effect upon one type of growth which is exactly the reverse of their effect upon another type of growth, and, furthermore, that as a general rule the effect of cholesterol upon a given type of growth is opposite to that exerted by lecithin upon the same type of growth.

<sup>1</sup> Robertson, T. B., *Arch. Entwicklungsmech. Organ.*, 1913, **xxvii**, 497

<sup>2</sup> Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, **xvii**, 344

<sup>3</sup> Browder, A., *Univ. California Publications, Physiology*, 1915, **v**, 1

<sup>4</sup> Bain, W., *Lancet*, 1912, **i**, 918

<sup>5</sup> King, H. D., *Biol. Bull.*, 1907, **xiii**, 40    Johnson, M., *Univ. California Publications, Zoology*, 1913, **xi**, 53

<sup>6</sup> Robertson, *J. Biol. Chem.*, 1916, **xxv**, 635

<sup>7</sup> Robertson and Burnett, *J. Exp. Med.*, 1913, **xvii**, 344    Burnett, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1913, **xi**, 42, 1914, **xii**, 33



The effects of these substances when administered by mouth upon the third growth cycle in mice (the growth subsequent to 4 or 5 weeks of age) have been discussed in the two preceding articles of this series. We have sought to extend these results by investigating the effects of lecithin and cholesterol upon the growth during the latter 2 weeks of the first growth cycle and the 1st week of the second growth cycle; that is, upon the growth of mice during the first 3 weeks succeeding birth. The young during this period, or at all events during the first 2 weeks of this period, are dependent upon the mother for their nutrition.<sup>8</sup> As the hypodermic administration of these substances to such young animals presents very great technical difficulties and the necessary manipulations might exert an uncertain effect upon the welfare of the animals, we have sought to influence the growth of the young through administration of lecithin or cholesterol by mouth to the mothers, fully recognising, however, that the interpretation of the results obtained is complicated by the possibility that the quantity and quality of the mother's milk may be directly affected by these substances, and that the growth of the young may in this way be indirectly affected, independently of direct transmission of the substances to the young through the medium of the mother's milk.

### *Methods.*

Twenty-five litters of mice were divided into three groups, A, B and C. The division was made at the birth of the litters, the litters being alternated so that the first litter came in group A, the second in group B, the third in group C, and so on. In this way the groups obtained consisted of nearly equal numbers of initially similar animals. The litters were kept in separate cages during the period of the experiment and the mother was supplied with an abundance of rolled barley and water, and occasionally with fresh lettuce leaves. In addition to this the control animals (group A) each received daily 1 cc. of mixed yolk and white of egg, another group of animals (B) received the same amount of egg mixture to which, however, were added 100 mg. of egg lecithin, prepared in the manner described in the preceding article,

<sup>8</sup> Robertson, *J. Biol. Chem.*, 1916, xxiv, 373.

while the third group received the same amount of egg mixture to which were added 100 mg. of Merck's cholesterol. The animals were weighed daily or every 2nd day to the nearest cg., each mouse in the litters being weighed separately. The litters were all kept in the same room and under identical conditions.

## RESULTS.

The results obtained are shown in Table I and depicted graphically in Figs. 1 and 2. It will be seen that the administration of egg lecithin to the mother produced a slight and nearly uniform retardation in the growth of the young, in keeping with its effect upon the later growth of mice when administered by mouth.<sup>9</sup> The administration of cholesterol to the mother, however, resulted in a much more profound retardation of the growth of the young,

TABLE I.

Age.	Normal.		Lecithin-fed		Cholesterol-fed	
	No weighed	Average weight	No weighed	Average weight.	No weighed	Average weight
days		gm.		gm		gm
Birth.	48	1.47	45	1.27	47	1.30
1	40	1.40	21	1.70	30	1.61
2	26	1.80	11	1.85	39	1.75
3	24	2.27	19	1.96	32	1.98
4	19	2.70	29	2.49	19	2.37
5	19	2.86	27	2.83	30	2.59
6	29	2.60	16	2.85	25	2.83
7	18	2.99	34	3.24	26	3.19
8	30	3.80	27	3.33	25	3.58
9	25	3.84	21	3.71	21	3.60
10	29	4.13	19	4.01	21	3.84
11	26	4.67	21	4.13	18	4.00
12	37	4.50	23	4.30	26	4.14
13	23	4.77	11	4.80	18	4.21
14	26	5.09	27	4.81	26	4.36
15	32	5.51	16	4.62	21	4.25
16	25	5.50	19	5.10	23	4.38
17	26	5.67	22	5.51	14	4.54
18	28	6.51	12	6.17	15	4.61
19	18	7.19	20	6.28	25	5.36
20	25	7.59	20	6.62	25	6.02

<sup>9</sup> Robertson, *J. Biol. Chem.*, 1916, xxv, 647.

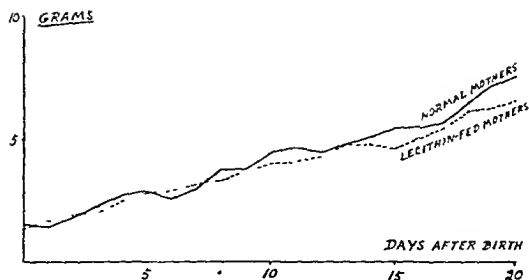


FIG. 1. Comparison of the growth of mice suckled by normal mothers with that of mice suckled by mothers fed with lecithin derived from egg yolk.

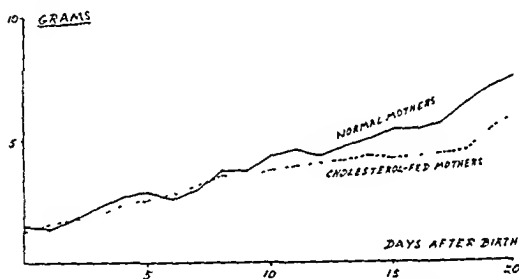


FIG. 2. Comparison of the growth of mice suckled by normal mothers with that of mice suckled by mothers fed with cholesterol.

an effect which was manifestly determined by the stage of development of the animals, for prior to the 9th day very little if any retardation of growth occurred, but thereafter, until the termination of the period of lactation on the 20th day after birth, accumulative and very decided retardation of the growth of the young was observed, with the result that by the 20th day after birth the sucklings of the cholesterol-fed mothers were 1.57 gm. or 21 per cent lighter than the young of the normal group. It would appear that cholesterol, when administered in this way, brings about retardation of the latter portion of the first and the initial portion of the second growth cycle,<sup>8</sup> but whether this effect is attributable to transmission of excess of cholesterol to the young through the medium of the mother's milk or to interference with the quantity or quality of milk supplied, our results do not as yet permit us to decide.

## SUMMARY.

1. The administration of 100 mg. of egg lecithin per day by mouth to the mother slightly retards the growth of suckling mice.

2. The administration of 100 mg. of cholesterol per day by mouth to the mother causes a very marked retardation of the growth of suckling mice between the 9th and 21st days after birth.

3. Our experiments do not enable us to decide whether these actions represent the direct effect of lecithin and cholesterol upon the growth of sucklings or only an indirect effect due to interference with the supply of milk.



# AN IMPROVED HASSELBALCH HYDROGEN ELECTRODE AND A COMBINED TONOMETER AND HYDROGEN ELECTRODE, TOGETHER WITH RAPID METHODS OF DETERMINING THE BUFFER VALUE OF BLOOD.\*

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### 1. The Two Compartment Hydrogen Electrode.

After experimenting for a year with hydrogen electrodes based on the form designed by Michaelis, the senior author attempted to modify Hasselbalch's electrode so as to eliminate the danger of loss of  $\text{CO}_2$  during transfer of solution. The electrode vessel (Fig. 1) has two compartments, the smaller of which contains the electrode proper. Hydrogen is shaken with a portion of the solution in the large compartment and then passed through the large middle stop-cock into the smaller compartment containing another portion of the solution, where the reading is made. Stop-cock *a* is not greased and conducts electrolytically while closed. Since the hydrogen is brought to an equilibrium with the first portion of the solution, it does not appreciably remove  $\text{CO}_2$  from the second portion. The lower the buffer value of the

\* The Apparatus was bought out of the Research Fund of the Graduate School.

solution, the larger the large compartment should be. If the two compartments are the same size, fairly accurate readings may be made on blood, but if the samples are not exceedingly small it is better to have the ratio of the compartments as in Fig. 1. A demonstration of the reliability of this electrode was made by charging it with blood serum and making a succession of readings from the end of 5 minutes until the end of 48 hours; they were the same within 1 millivolt. The potentiometer and Weston cell were compared with ones recently calibrated by the Bureau of Standards.

A description of the technique of determinations on blood will suggest the precautions necessary for any solution. The blood

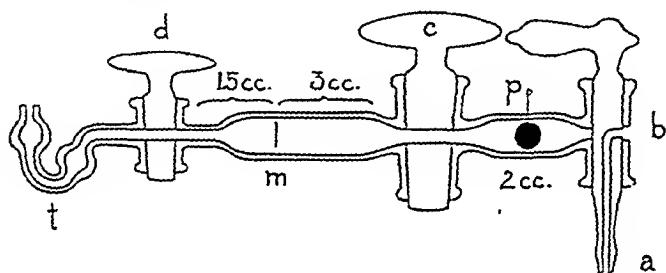


FIG. 1. Two compartment hydrogen electrode. Hydrogen is shaken with one portion of the blood in the large compartment and passed into another portion of the blood in the small compartment for the measurement of the electrode potential.

is collected as described by McClendon (1916 a), and the defibrinated blood or serum used, or it is passed directly into the electrode by means of a rubber tube connected to the needle or cannula in the blood vessel. In the latter case, the electrode must contain some hirudin or isotonic, neutralized sodium oxalate. The dilution of blood with an equal volume of a neutral solution of low buffer value has been shown by Michaelis, and more recently by Corral, to have no perceptible effect on the reaction.

In order to obtain absolute values, the electrode must be cleaned with potassium bichromate in  $\text{H}_2\text{SO}_4$  and replatinized a few seconds by removing the stop-cock *a* and inserting a platinum wire anode through the opening *b* (Fig. 1). It is then thor-

oroughly rinsed with distilled water (and the stop-cock *a* lubricated with serum or KCl and glycerol) and filled with pure hydrogen; a drop of water in the trap *t* serving to prevent the backward diffusion of air until all stop-cocks are closed. The rubber tube admitting the blood is connected to *a*, and the air in it allowed to pass out at *b*, after which this stop-cock is turned so as to admit the blood into the electrode, when the other stop-cocks are opened. The blood is allowed to enter until it reaches the mark *m*, the apparatus then containing 5 cc. of blood and 1.5 cc. of hydrogen.

All the stop-cocks are closed and the apparatus is shaken or inverted 200 times. The middle stop-cock is opened and the apparatus tapped or swung so as to cause the hydrogen to pass into the smaller compartment by displacing part of the blood. This backflowing blood helps to complete the equilibration of the hydrogen in regard to  $\text{CO}_2$ . The smaller compartment now contains 1.5 cc. of hydrogen and 0.5 cc. of blood, and is shaken or inverted 200 times; and the stop-cock at *a* is immersed in the KCl solution connected with the calomel electrode. The connection is completed by hooking a wire in the platinum loop *p*, the wire being bright and weighted sufficiently to maintain a good contact.

If there is but little oxygen in the blood, the definitive reading should be obtained immediately (provided it is the same temperature as the calomel electrode), but if it contains much oxygen-hemoglobin the reading will be too low, and will slowly rise as the oxygen combines with the hydrogen in the platinum black. If more than 15 minutes are allowed to elapse, the electrode should be shaken in order to bring a fresh layer of blood into contact with the platinum before another reading. It is not necessary to shake continuously, as recommended by Hasselbalch, but continuous shaking hastens the disappearance of the oxygen. If much hydrogen disappears by combination with the oxygen, it is necessary to restore atmospheric pressure by momentarily opening the stop-cocks *c* and *d*, but this cannot be done more than once without danger of admitting oxygen from the air.

Since convenience and accuracy depend on the details of the construction of the electrode, a few words as to its manufacture may be permitted. If platinum is used for the electrode proper,



it must be exceedingly thin so as not to absorb an appreciable amount of hydrogen. Gold is much better, but must be attached to a platinum wire fused through the glass. Platinum may be welded to platinum at a white heat, but gold melts at this temperature. It is better to melt a drop of gold on the end of the platinum wire and hammer this drop (after cooling) to the gold disc. In doing this, a gold wire or scrap of foil is wrapped around the end of the platinum wire and held in the flame until it is just melted. If held in the flame longer, the gold will amalgamate with the platinum, forming a brittle alloy.

The bore of stop-cock c should be at least 3 mm., as the larger it is the more easily the hydrogen is caused to pass into the small compartment.<sup>1</sup>

## *2. The Hydrogen Ion Concentration of Blood and Serum.*

It is stated by Hasselbalch and others that serum is more alkaline than blood. Hasselbalch found that the less oxygen blood contained, the more alkaline it appeared to be when measured by the hydrogen electrode. Since hemoglobin may carry a large store of oxygen, the discrepancy between the reaction of blood and serum is apparently due to faulty technique. Although it is theoretically impossible for the mere proximity of the corpuscles to change the reaction of the plasma, and Michaelis claims that coagulation does not affect the reaction, it seemed advisable to test this question experimentally. The result was that the reaction of serum and defibrinated blood from the same source and without loss of  $\text{CO}_2$  is the same. This tells nothing concerning the reaction of the interior of the corpuscles or the effect of laking on the reaction of the blood, which will be considered in a later paper.

Hasselbalch states that the  $P_H (= -\log H^+)$  of blood is 0.17 lower at  $37^\circ$  than at  $19^\circ$ , and Michaelis states that  $P_H$  determinations at  $37^\circ$  are 0.21 lower than the average of determinations on different persons at room temperature. Since Corral and others have not observed high temperature coefficients for the hydrogen

<sup>1</sup> The best electrodes were blown by A. S. Jones, 62 Alexander St., Princeton, N. J., who also made the stop-cocks, and the tonometers to be described.

ion concentration of various buffer mixtures, it seemed strange that the blood should be an exception. In order to test this, two saturated KCl calomel electrodes were compared at the same temperature and found to be alike. One of them was placed in a thermostat at  $37^{\circ}$  and allowed to remain 3 days, since Wolff found it took a long time to come to equilibrium. Blood and serum were tested at  $23^{\circ}$  and at  $37^{\circ}$  against these calomel electrodes. The difference in the  $P_H$  at the two temperatures was not greater than 0.02 and was therefore within the limit of experimental error. The same was found true of  $\text{NaHCO}_3$  solutions. This experiment was repeated with 0.1  $N$  KCl calomel electrodes. The  $P_H$  was lower at the higher temperature, but the difference did not exceed 0.01 to 0.07 when corrected for the increased vapor tension of water.

The reason for using two types of calomel electrodes is that the result depends on the value of the *r. m. r.* of the calomel electrode against the normal hydrogen electrode, as calculated by different investigators. In such calculations the hydrogen ion concentration in the hydrogen electrode is determined by electric conductivity or other indirect means. Ellis observed that the  $H$  ion concentration of  $\text{HCl}$ , even at 0.005  $N$ , is different, when calculated from conductivity data, from the value obtained by electrode potential. For this reason the results obtained on blood at different temperatures are not to be considered absolute, but merely reproducible by using the ordinarily accepted data, as given by McClendon (1916 a, Fig. 5) and Michaelis.

Since the viscosity of water decreases with rise of temperature the dissociation of acids, calculated from conductivity data, is erroneous unless these data are corrected for viscosity. Owing to the increased dissociation at higher temperature, the  $P_H$  of pure water is about 0.3 less at  $37^{\circ}$  than at  $18^{\circ}$ , but the  $P_H$  of an alkaline solution should be greater at the higher temperature. We found the  $P_H$  of  $N$   $\text{NaHCO}_3$  to be 7.9, the  $P_H$  of 0.1  $N$  solution, which is dissociated (hydrolytically) to a greater extent, to be 8.3. The latter result confirms that of Auerbach and Pick. Apparently the effect of rise in temperature increasing the dissociation of water and thus decreasing the  $P_H$  is partly counterbalanced by the greater dissociation of alkaline salts, increasing the  $P_H$ , so that the reaction of the blood remains nearly the same when

measured by means of the hydrogen electrode at different temperatures. The dissociation of water and hence the hydroxyl ion concentration in the blood increase with rise in temperature; hence the blood becomes more alkaline with rise in temperature, as pointed out by Höber.

Hasselbalch has shown that the  $P_H$  of arterial blood (*i.e.*, at alveolar  $CO_2$  tension) is remarkably constant. On the contrary, Menten and Crile claim that the blood returning from different organs shows great differences in  $P_H$ . The unpublished determinations which the senior author made for Dr. Uhlrich, using electrodes previously described, showed variations in the  $P_H$  of venous blood. The average  $P_H$  of venous blood is less than 0.05 lower than that of arterial blood, and smaller fluctuations in the  $CO_2$  content must necessarily have less effect on the reaction. The records in the literature of the  $CO_2$  content of blood returning from various organs are very discordant. According to Hill and Nabarro, activity of an organ sometimes reduces the  $CO_2$  content of its venous blood because the effect of increased metabolism is overcompensated by vascular dilatation. Perhaps the chief cause in the variations in the  $P_H$  of venous blood is the variation in the blood flow, and hence stasis attending the collection, which is more or less unavoidable in the human subject, is a real source of error.

It has been thoroughly established that changes in the H ion concentration of the blood affect the respiratory center, but for those who hope to detect a change in the reaction of the blood during dyspnea, a little calculation may be of interest. Haldane and Priestley observed that a rise of 0.2 per cent in the  $CO_2$  of the alveolar air doubles lung ventilation. The change in the H ion concentration of the blood caused by a rise in the  $CO_2$  tension of 0.2 per cent of an atmosphere is far within the limits of error of the gas chain method as applied to blood. Hence measurable changes in the  $P_H$  of arterial blood mean changes in the threshold for stimulation of the respiratory center as in shock or after drugs.

The respiratory center is apparently not so sensitive to the infusion of HCl into the blood, but this may be due to a difference in permeability and concentration gradient. Since  $CO_2$  is produced by the respiratory center, an increase in this gas in the blood

would decrease the outward diffusion from the center, which would become less alkaline. A mere increase in the H ion concentration of the blood would have much less effect on the diffusion of  $\text{CO}_2$ , since only a minute fraction of the latter is dissociated. If acid were injected until free acid appeared in the blood, some would diffuse into the respiratory center. Judging by experiments on other cells, fatty acids should diffuse in faster than mineral acids, and hence be more effective in increasing respiration, as is actually found to be the case.

When HCl is added to blood *in vitro*, its content of free  $\text{CO}_2$  is increased, but it is incorrect to suppose that the same final result is obtained by infusion of HCl. In the latter case the respiratory center is stimulated and the  $\text{CO}_2$  eliminated, so that in a short time the  $\text{CO}_2$  tension of the blood is below normal. Since the content of the blood in free  $\text{CO}_2$  varies directly with the  $\text{CO}_2$  tension, the infusion of HCl reduces the free  $\text{CO}_2$  in the blood. The reduction is not sufficient, however, to maintain the normal reaction of the blood, as first shown by Szili.

Owing to the impossibility of measuring the H ion concentration of the respiratory center itself, all factors with a possible influence on it should be studied. For this reason attention is called, in the next section, to the buffer value.

The normal  $P_{\text{H}}$  of venous blood seems to fluctuate about 7.5, and that of arterial blood to be a little higher, but the difference is not usually measurable. The number of determinations have been too few, however, to separate individual variations entirely from errors, and no absolute values can be given.

### 3. The Combined Tonometer and Hydrogen Electrode.

A combined tonometer and hydrogen electrode was described by Peters, but the large rubber stopper rendered it objectionable. The form shown in Fig. 2 has been found convenient and reliable. The gold disc with platinum wire *p* is sealed in the 2 cc. compartment, and the stop-cock *c* is lubricated with a conducting solution and immersed in the KCl trough so that this part of the apparatus can be used as a hydrogen electrode in the same manner as the form shown in Fig 1. The description of the manipulation of the apparatus for blood is the same as for any other fluid. The blood is admitted at *a* until it completely fills the

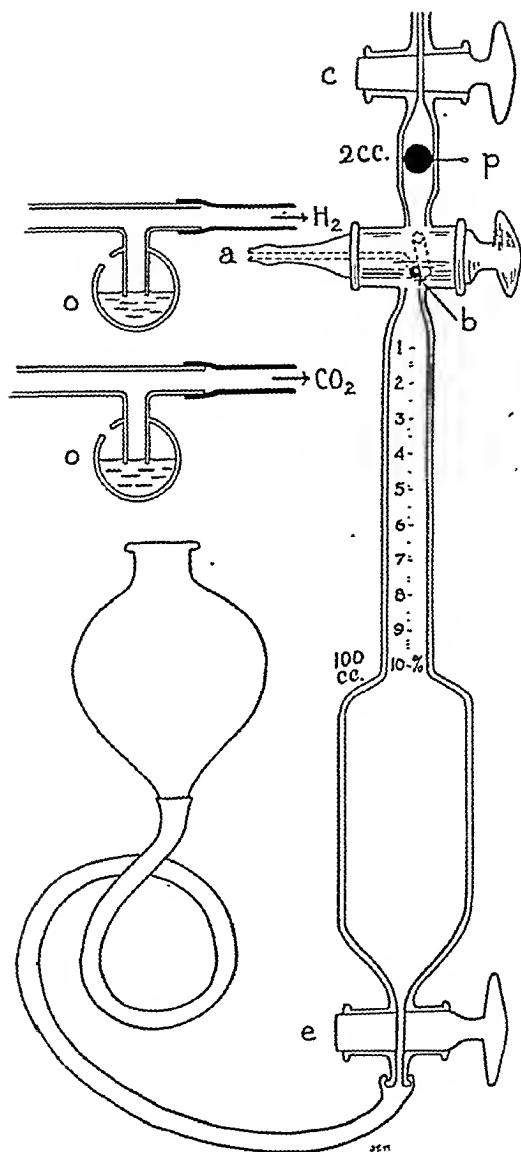


FIG. 2. Combined tonometer and hydrogen electrode. Blood is passed from the 2 cc. compartment into the 100 cc. tonometer and back into the 2 cc. compartment, where the electrode potential is determined.

2 cc. compartment, and the stop-cocks are closed. The apparatus is set up vertically and a rubber tube from a mercury funnel attached to the lower end *c*. By raising the funnel, the 100 cc. tonometer is filled with mercury which forces the air out at *a*. The tubes from the hydrogen and  $\text{CO}_2$  generators are provided with overflows *o* to keep these gases at constant and approximately atmospheric pressure. The  $\text{CO}_2$  is admitted at *a*, and the stop-cock *a* turned so as to force out the contained air at the side opening *b*, and then turned back so as to allow the  $\text{CO}_2$  to enter the tonometer when the funnel is lowered. When the required per cent of  $\text{CO}_2$  is read off on the graduations of the tonometer, the stop-cocks *c* and *a* are closed, the hydrogen tube is connected to *a*, the air washed out at *b*, the tonometer filled with this gas, and *c* closed. The stop-cock *a* is so turned as to connect the 2 cc. chamber with the tonometer by means of a hole that is at least of 3 mm. bore. By tapping or swinging the apparatus, the blood is shaken down into the tonometer, which is rotated on its long axis (placed horizontally) by means of a rubber band passed around a revolving axle above it. About 0.5 cc. of the blood is shaken back into the 2 cc. chamber, *c* immersed in the KCl solution, *p* connected to a wire, and the reading taken in the usual manner.

The remainder of the blood in the tonometer is forced into the 2 cc. chamber by means of mercury, and the second  $\text{CO}_2$  mixture made in the tonometer in the same manner as the first. The same sample of blood will do for a series of determinations provided the mercury is pure. Before using the apparatus, it should be prepared in the same manner as the electrode shown in Fig. 1.

#### 4. Charts for Finding the Buffer Value of Blood and Serum.

In a previous paper (McClendon, 1916 a) the buffer value was indicated by the difference between the  $P_n$  at 3 per cent and at 10 per cent  $\text{CO}_2$  tension. The difference in this index of the buffer value for different bloods is so small as to make the charts shown in Fig. 3 desirable as aids in its determination.<sup>1</sup> They will at

<sup>1</sup> The quantity of data used in the making of these charts is not as large as might be desired, but the fact that the junior author cannot continue the work makes it necessary to publish them in their present form.

least serve as guides in the construction of similar charts by investigators engaged in similar work. The data on blood are not in disagreement with the limited observations of Hasselbalch, but no comparative data on serum were found in the literature. The  $P_H$  values found by Hober are too low.

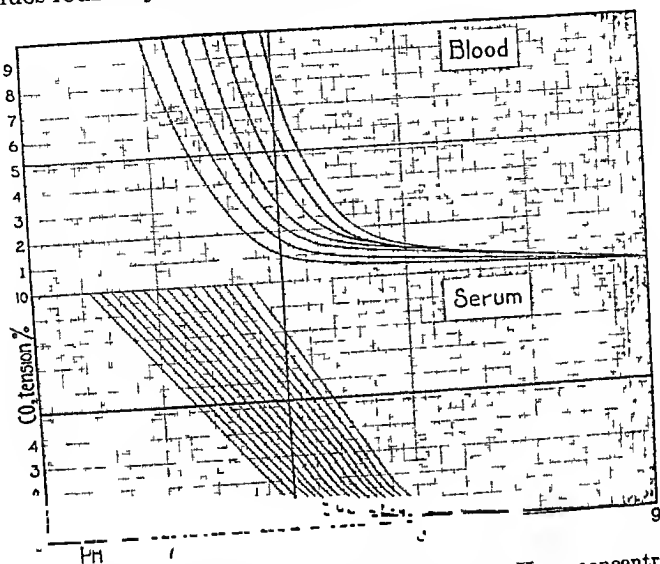


FIG 3 Curves showing the change of  $P_H$  ( $= -\log H$  ion concentration) with change of  $CO_2$  tension. On the ordinates are measured the percentages of an atmosphere in moist  $CO_2$ , and these may be reduced to mm. of dry  $CO_2$  by multiplying by 72, since the tension of water vapor was 20 mm. and the barometric pressure 740 mm. At high altitudes all calculations should be made in mm, but near the sea level the error is not appreciable if the calculations are made in percentage of an atmosphere, as a slight change in  $CO_2$  tension makes a much smaller change in  $P_H$ . The electrode potential calculated for 760 mm of dry hydrogen was used in determining the  $P_H$ .

Since the  $P_H$  of arterial blood both in health and disease is remarkably constant (about 7.5) the curve in the chart corresponding to any sample of blood or serum may be found by one observation. If the alveolar  $CO_2$  tension by the Haldane method is determined, the curve is the one passing through the intersection of this  $CO_2$  tension and the  $P_H = 7.5$  line. If the  $P_H$  at any known  $CO_2$  tension is determined, the curve is the one

at the intersection of these  $P_{H_2}$  and  $CO_2$  tension coordinates, and the intersection of this curve with the  $P_{H_2} = 7.5$  line shows the alveolar  $CO_2$  tension (unless the respiratory center is abnormal as in shock or after caffeine or morphine).

The alveolar  $CO_2$  tension by Haldane's method is practically the same as that of the arterial blood. The alveolar  $CO_2$  tension by Plesch's method may, by proper manipulation, be made to show the average  $CO_2$  tension of the venous blood. The normal arterial  $CO_2$  tension of the arterial blood is about 5.2 per cent, and of the average venous blood about 6 per cent of an atmosphere, or about 16 per cent higher than arterial. Higgins used a modified Plesch apparatus, in which the entire air was rebreathed four times (about 20 seconds), and obtained values about 20 per cent higher than by Haldane's method. It would be interesting to know whether such a ratio is constant even when cases of extremely low  $CO_2$  tension are included. If such were the case, the true alveolar  $CO_2$  tension might be calculated from the results by the Plesch method.

The striking fact shown by the charts is that the buffer value of the blood or serum depends on its  $CO_2$  tension. Below 1 per cent  $CO_2$  tension, the buffer value is almost nil. Above 5 per cent  $CO_2$  tension, the buffer value of the blood is almost infinite, while that of the serum is somewhat less. Therefore, the buffer value of the blood of a patient with alveolar  $CO_2$  tension equal to less than 1 per cent of an atmosphere, is so low as to make it probable that great local differences in the reaction of the blood exist in the body. Such local variations probably have a great deal to do with the dyspnea of many patients. The acid produced in the respiratory center itself may be very poorly neutralized by the blood, and temporary increased breathing must result.

#### *5. The Indicator Method for Determining the Buffer Value.*

At the request of Dr. Rowntree we have calibrated his indicator method for determining the reaction of blood. Phosphates of known water content were used to make solutions according to Sørensen's directions, and portions of the same phosphates sent to Hynson, Westcott and Company for the manufacture of



sealed tubes of standard solutions colored with phenolsulfonephthalein. These tubes were then compared with similar ones calibrated by the gas chain method. The only difficulty experienced was in obtaining "nonsol" tubes of exactly 1 cm. bore, a small divergence from which caused an appreciable error. Blood was tested by the gas chain method and then dialyzed 7 minutes against isotonic, neutral (tested) NaCl solution in stoppered "nonsol" tubes and tested with phenolsulfonephthalein. If the transfer and other manipulations were sufficiently rapid so as not to lose  $\text{CO}_2$  appreciably into the air, and the tubes were of exactly 1 cm. bore, the results did not disagree with those of the gas chain method. The latter could be read, however, to one more decimal place. In attempting to prevent  $\text{CO}_2$  loss by a layer of oil, it should be remembered that  $\text{CO}_2$  is more soluble in oil than in water, and the oil merely lessens convection (by its non-miscibility).

The following method for determining the buffer value clinically is suggested. 3 or more cc. of blood mixed with hirudin or neutralized oxalate are introduced into a 100 cc. tube with openings at each end of 1 cm. bore provided with rubber tubes and pinch-cocks. The blood is introduced into this tube and the breath of a person of normal (previously tested) alveolar  $\text{CO}_2$  tension blown through it, the pinch-cocks being closed after the last breath is forcibly expelled. The tube is rotated 10 minutes and the reaction of the blood determined by the indicator-dialysis method. The intersection of the line of this  $P_H$  with the 5.2 per cent  $\text{CO}_2$  tension line in the chart will show the curve corresponding to the blood sample. The intersection of this curve with the 7.5  $P_H$  line will show the alveolar  $\text{CO}_2$  tension of the patient (the error will be small except in case of shock or after certain drugs).

#### 6. The Indicator Method for $P_H$ of Stomach Contents.

Stomach contents were dialyzed through collodion sacs as first tried by Fowler, Bergeim, and Hawk. The buffer value varies directly with the  $P_H$  and is very low for high acidity, in which case the volume of the stomach contents in the sac should far exceed that of the 1 per cent NaCl solution in the tube containing the sac. For low acidity, Davidsohn has shown that dilution affects the  $P_H$  very little.

The dialysate was compared with the published colored chart (McClendon, 1916 b), using the indicators noted in the chart. A calibration of this method showed that it was significant for the integral part of the  $P_{H}$ . Scaled tubes of standard solutions with some of the indicators were made also but, unfortunately, methyl violet, the most useful indicator, fades in very acid solutions. Manveine was not obtainable, but it is hoped that it would not fade so rapidly. Neutral red shows a striking change to blue in very acid solutions, but the acidity required is higher than is usually the case with stomach contents.

In conclusion, thanks are due to Dr. J. W. Northington and Miss Swift for aid in the collection of blood samples.

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